Differentiation in Pseudomonas Aeruginosa Biofilms

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Sünje Johanna Pamp

Ph.D. thesis
BioCentrum-DTU
Technical University of Denmark

DTU
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PhD thesis

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by

Sünje Johanna Pamp

October 2007

BioCentrum-DTU
The Technical University of Denmark

There are a number of levels of biological organization and each level offers unique problems and insights, and each level finds its explanation of mechanism in the levels below, and its significance in the levels above.

George A. Bartholomew, 1964

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Cover illustration: *Pseudomonas aeruginosa* cells representing different phenotypic cell types: *yellow* - motile cells, *blue* - non-motile cells, *green* - metabolic active cells, *red* - dead cells.

Summary

The majority of microbial cells on Earth live in intimate relationship with other microbial cells to form multicellular communities, also called biofilms. Most biofilms can be regarded as beneficial, however, occasionally they can be harmful such as in cases where they are the cause of persistent infections. One challenge is to unravel processes involved in the development of biofilms, and learn about interactions taking place between the participating biofilm cells, and interactions taking place between the biofilm cells and their environment. Consolidated knowledge derived from research work addressing these issues will enhance the understanding of the microbial biofilm mode of living, and reveal new paths that might help to develop strategies to prevent and treat persistent infections.

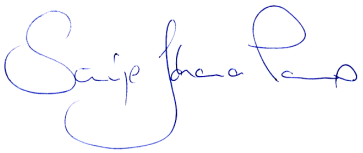
The objective of this experimental thesis work was to address the differentiation of a biofilm to gain insight into mechanisms involved in biofilm development, structural composition, and to learn about interactions between biofilm cells and between biofilm cells and their environment. As model-system was used flow-chamber-grown mushroom-shaped *P. aeruginosa* biofilms. In a first study, the role of excreted biosurfactants on differentiation of the mushroom-shaped biofilm was investigated. It was found that the biosurfactants impact on i) differentiation of the stalk-forming cell subpopulation, potentially by facilitating cellular aggregation, and ii) differentiation of the cap-forming cell subpopulation, potentially by facilitating cellular migration. Further experiments showed that flagella are required for differentiation of the cap-forming subpopulation, as a flagella mutant is impaired in colonization on top of the stalk-forming subpopulation. Additionally, the chemotaxis cluster IV was found to be involved in modulating the shape of the cap-forming subpopulation, as the cap formed by various cluster IV-mutants are differentially shaped compared to the cap formed by the wild type.

A previous study had shown that exposure of *P. aeruginosa* biofilms to the antimicrobial peptide colistin preferentially induced cell death in the cells of the stalk-forming subpopulation, whereas the cap-forming subpopulation survived the treatment under involvement of the *pmr*-operon. In the context of this thesis work it was addressed in more detail, why this particular subpopulation was able to develop colistin-tolerance, in contrast to the stalk-forming subpopulation. The results provide evidence that the cap-forming subpopulation is composed of metabolic/physiological active cells, which are able to adapt to colistin by developing tolerance via a mechanism involving the *pmr*-operon and *mexAB-oprM*-genes. In contrast, the stalk-forming subpopulation, exhibiting low metabolic activity, is not able to induce tolerance and is therefore killed by colistin. Other membrane-targeting compounds, such as the antimicrobial peptide novispirin G10 or the antiseptic chlorhexidine gluconate, were found to induce cell death specifically in the cells of the stalk-forming subpopulation as well. Furthermore, evidence was provided that the metabolic active cells of the cap-forming subpopulation are susceptible to conventional antimicrobial compounds, such as ciprofloxacin, tetracycline and imipinem. Intriguingly, systematic combined antimicrobial treatments, simultaneously targeting the two distinct physiological subpopulations (cap & stalk), were found to eradicate almost all cells in the biofilm.

Together, the results obtained from this thesis work reveal different factors that impact on biofilm development and differentiation of distinct subpopulations in this *P. aeruginosa* model system. Moreover, the data provide evidence that upon adverse changes in environmental conditions a particular subpopulation of cells can obviously sense and adapt to these conditions. Finally, knowledge about the physiology of the biofilm cells revealed a strategy to almost entirely eradicate the biofilm. These findings might have implications for the development of systematic treatment strategies in medical settings.

Preface

This dissertation is submitted as partial fulfillment of the requirements to obtain the Ph.D. degree at The Technical University of Denmark (DTU). This work was carried out at BioCentrum-DTU in the period from August 2004 to October 2007 with Associate Professor Tim Tolker-Nielsen as supervisor. As graduate student I was associated to the European Graduate School '*Pseudomonas*: Pathogenicity and Biotechnology', and to the 'Større tværgående forskergrupper: Microbial Opportunistic Pathogens'. This work was funded by the Danish Agency for Science, Technology and Innovation and The Technical University of Denmark.



Sünje Johanna Pamp

Charlottenlund, October 2007

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I would like to express my gratitude to everyone who supported me during the last years and from whom I could learn different aspects of scientific life.
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Janus A. J. Haagensen is acknowledged for introducing me into the art of confocal laser scanning microscopy and cultivation of flow-chamber biofilms. I thank you also for your assistance once in a while outside your normal working hours in cases of technical trouble with the microscope, and for proof reading my thesis. I am very grateful to *Paula Ragas* for supporting me on the 'pilG-project'. It was a great pleasure to work together with you, and to learn more about molecular cloning and DNA microarray technology. The cloning expert *Morten Gjermansen* is acknowledged for supporting me with the construction of four mutant strains, which I would not have been able to make by myself in such a short time. I was fortunate to share the office with some wonderful people, *Morten G.*, *Mikkel Klausen* and *Inger Olesen*, some who stayed longer and some who sought new challenges elsewhere. Thank you for the funny moments, for listening, for your help and special thanks to *Morten G.* for enforcedly accepting all the plants I brought into our office☺. Thank you, *Claus Sternberg*, for being always there in cases of acute needs in particular with the various technical equipments. I am very grateful to the people who are always happy to help with useful advices, expert knowledge and providing with the many essential 'small-big' things, thank you *Tove Johansen*, *Anne K. Nielsen*, *Birthe J. Jørgensen*, *Lisser St. Clair-Norten*, *Hanne Christensen*, *Anita Sørensen*, *Brian Howard*, *Lis Hansen*, and *Lone Hansen*. Special thanks to *Mette E. Skindersø* for the superb candy-shop - I would not have survived all the long days without. Thank you also for your positive attitude, all the social ice-cream events, and that we could share phases of up and down as part of our graduate student life. Thank you, *Morten Harmsen*, for passing by the office, sitting down in my couch with a cup of coffee in your hand and for all the chats. Thanks for teaching me, presumably unwittingly, and thanks for your support during the Biological Sequencing Analysis Course. I am grateful that I had the chance to get to know *Lars Jelsbak* - thank you that we could exchange thoughts. Thanks to *Kim Barken*, *Liang Yang*, *Thomas Bjarnsholt,* and *Helle K. Johansen* for fruitful collaborations. Thanks to *Anders Folkesson* for all the chats about antimicrobial peptides and helpful advice. It has been a great pleasure to also spend time with and learn from *Susse K. Hansen, Thomas B. Rasmussen*, *Carsten Matz*, *Susse Grathe*,

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I thank all members of the *European Graduate School 'Pseudomonas: Pathogenicity and Biotechnology*', and the '*Større tværgående forskergrupper*: Microbial Opportunistic Pathogens' for creating such inspiring environments. I am very grateful to the organizers and participants of the '*Analytical and Quantitative Light Microscopy*' course at the MBL in Woods Hole, USA, for an excellent course and memorable time.

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Publication list

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Pamp, S. J., Gjermansen, M., Johansen, H.K. and Tolker-Nielsen, T. Tolerance to the Antimicrobial Peptide Colistin in *Pseudomonas aeruginosa* Biofilms is Linked to Metabolically Active Cells, and Depends on the *pmr* and *mexAB-oprM* Genes. Submitted…….....Paper 3

Barken, K. B., **Pamp, S. J.**, Yang, L., Klausen, M., Givskov, M., Bertrand, J., Whitchurch, C., Engel, J., and Tolker-Nielsen, T. Roles of Type IV Pili, Flagella, and Extracellular DNA in Structural Development of *Pseudomonas aeruginosa* Biofilms. In preparation.....Paper 4

Pamp, S. J., Haagensen, J.A.J., Molin, S., and Tolker-Nielsen, T. A Distinct Subpopulation of Cells in *Pseudomonas aeruginosa* Biofilms can be Specifically Eradicated Using Antimicrobial Compounds that Cause Membrane Disruption. In preparation……..Paper 5

Not included in this thesis:

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Qin, Z., Yang, L., **Pamp, S. J.**, Qu, D., Givskov, M., Molin, S. Tolker-Nielsen, T. *Pseudomonas aeruginosa* Extracellular Products Inhibit Staphylococcal Growth and Disrupt Established Biofilms by *Staphylococcus epidermidis*. Submitted.

van Gennip, M., Christensen, L. D., Mikkelsen, P. J., Jensen, P. Ø., **Pamp, S. J.**, Phipps, R., Moser, C., Tolker-Nielsen, T., Høiby, N., Pier, G. B., Givskov, M., and Bjarnsholt, T. Quorum Sensing Controlled Rhamnolipid Production is Crucial for *Pseudomonas aeruginosas* Ability to Evade the Immune Response in Three Different Animal Models of Infection. In preparation.

Contents

1 Introduction

This chapter aims at introducing the basic aspects and methodology underlying the objective of this thesis. At first, the role of microorganisms from the human perspective will be presented in a historical context. Thereafter the microbial biofilm mode of living and planktonic mode of living in nature will be depicted. At last, tools used to cultivate and study biofilms under laboratory conditions will be introduced.

1.1 Microorganisms – in historical context

Bacteria were the first life-forms on Earth and presumably will also be the last ones. They populate terrestrial and aquatic environments as well as the air and have established intimate relationships with animals and plants. Every process in the biosphere appears to be dependent on microorganisms. They convert key elements of life, such as carbon, nitrogen, oxygen and sulfur, into forms accessible to all other living matter (Madsen, 2005; Whitman *et al.*, 1998). It is assumed, that bacteria inhabit this planet for approximately 3.0 billion years (Cavalier-Smith *et al,* 2006), and consequently animals and plants have evolved in the presence of bacteria. The field of studies of microorganisms is a relatively young area, compared to zoology and botany, simply due to the small size of the objects to be studied and hence usually invisible for the naked eye. Microorganisms remained unseen, until Robert Hooke (1635-1703) looked through his compound microscope at some hairy fungi arising from putrefaction of vegetables, cheese, leaves and leather (e.g. Fig. 1a). Antonie van Leeuwenhoek (1632-1723) was the first who observed and recorded bacterial cells using his relatively simple microscope (Fig. 1b), which allowed a higher magnification compared to the microscope used by Hooke.

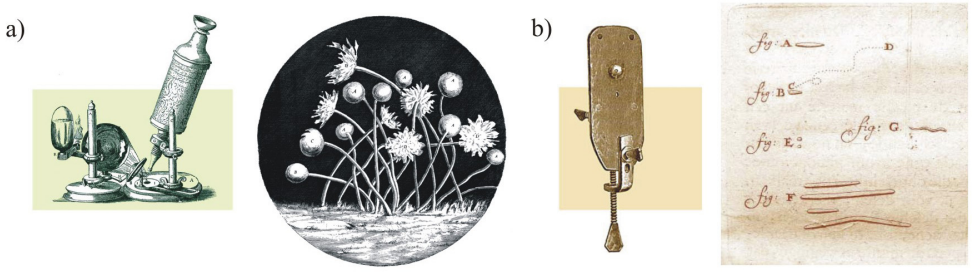

Fig. 1. The first microscopic observations of microorganisms. *a)* Robert Hooke was the first who published detailed 'descriptions of minute bodies made by magnifying glasses' in *Micrographia* (1664, Royal Society). A drawing of the handcrafted microscope he used to make observations for *Micrographia* is depicted to the left. To the right: A drawing made by Hooke from hairy fungi, which he observed on the leather-cover of a small book (Source: Micrographia/Project Gutenberg). *b)* Antonie van Leeuwenhoek was the first who published observations of bacteria. The handcrafted microscope, he made these observations with, is depicted to the left. To the right: A drawing made by Leeuwenhoek of bacteria (rods, motile rods, cocci, and spirilla), which he observed in a sample of tooth plaque. (Source: Dunn and Jones, 2004). Images are not shown to scale.

The discovery of microorganisms in relation to infectious diseases

Already ancient people recognized that some diseases were 'communicable' and that frequently those who recover from a particular disease were not attacked again. However, it was not until the 19th century, that Louis Pasteur (1822-1895) and Robert Koch (1843-1910) set the foundation for the field of microbiology through groundbreaking research work on the *germ theory of disease*. Pasteur disproved the *theory of spontaneous generation of life* through experiments involving unique designed swan-necked flasks. And as he was working on fermentation, he discovered that chemical changes can be caused by living organisms. Moreover, he developed vaccination against chicken cholera, anthrax, and rabies. Koch invented methods to purify and grow pure cultures of bacteria (including agar plates and the Petri dish) and generally improved bacterial methodology. He formulated four criteria, the *Koch's postulates*, designed as a standard proof to verify a causal relationship between a causative microorganism and a disease. Applying these criteria, he identified the etiologic agents of tuberculosis (*Mycobacterium tuberculosis*), cholera (*Vibrio cholera*), and anthrax (*Bacillus anthracis*) (Beck, 2000; Lederberg, 2000). Since then, increasing numbers of researchers systematically applied scientific analysis to learn about the nature of microorganisms.

The discovery of antimicrobial compounds produced by microorganisms

A major breakthrough in the field of modern medicine occurred with the discovery that microorganisms can produce molecules, which can kill other microorganisms. It was fortuity that Alexander Fleming (1881-1955) observed that a compound released by a colony of *Penicillium notatum* situated on an agar plate was able to inhibit growth of Staphylococci situated in close proximity (Fig. 2) (Fleming, 1929). Howard W. Florey (1898-1968) and Ernst B. Chain (1906-1979) were able to isolate the active compound penicillin and detected its excellent pharmacological and chemotherapeutic properties (Chain *et al.*, 1940). At the same time, they already report on "An enzyme from bacteria able to destroy penicillin" (Abraham and Chain, 1940). Nevertheless, since its discovery, penicillin has saved the lives of millions of people suffering from a great range of infectious diseases. Interestingly, already in 1946 Fleming noted that, "the administration of too small doses leads to the production of resistant strains of bacteria" (Amyes *et al.*, 2001). It was also Fleming, some years prior to the discovery of penicillin, who discovered the bacteriolytic activities of lysozyme he found in human tissues and secretions (Fleming, 1922).

Pioneering research work on a variety of natural product antibiotics was performed by Selman A. Waksman (1888-1973) and Rene J. Dubos (1901-1982), which led to the discovery of antibiotics, such as streptomycin, effective against various human pathogens e.g. *Mycobacterium tuberculosis* (Beck, 2000). Among the first described peptide antibiotics were gramicidin, tyrocidin, subtilin, bacitracin, and polymyxin, produced by bacterial species such as *Bacillus brevis*, *Bacillus subtilis*, and *Paenibacillus polymyxa* (Ainsworth *et al.*, 1947; Benedict and Langlykke, 1947; Hotchkiss and Dubos, 1940; Katz and Demain, 1977; Stansly *et al.*, 1947). Altogether, the discovery and subsequent increasing application of antimicrobial agents certainly affects human ecology, but also causes evolutionary changes in other living organisms on Earth (e.g. Palumbi, 2001). It can be assumed that microorganisms in nature produce an astonishing diversity of small compounds exhibiting biological activity, and that they also must constantly be exposed to and sense and respond to these compounds. However, remarkably little is known about the natural role of these small chemical products. Observations suggest that these molecules have various functions, such as that they serve inter- and intra-species signaling and communication or as chemical weapons (Firn and Jones, 2000; Yim *et al*., 2006).

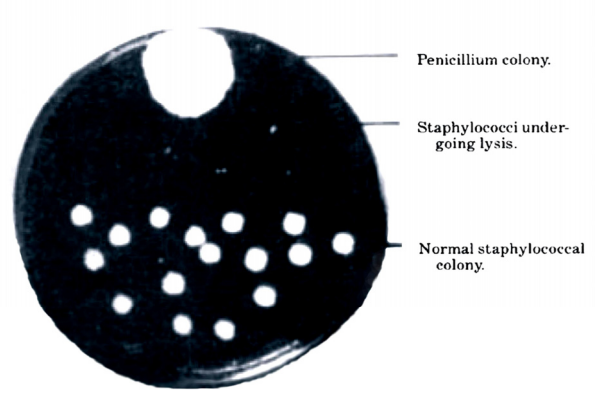

Fig. 2. The discovery of penicillin. Alexander Fleming noticed that when he left a culture plate with colonies of *Staphylococcus* on a bench for an extended period, a colony of a contaminating mould (*Penicillium*) had developed, and that growth of *Staphylococcus* colonies situated around the *Penicillium* colony was inhibited (see image) (Source: Fleming, 1929). Moreover, he carried out detailed investigations with culture fluids of *Penicillium* and found that it contained a compound, termed 'penicillin', that can inhibit growth of various bacteria, such as *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and *Corynebacterium diphteriae* (Fleming, 1929).

The biology of microorganisms

Empirical research work, supported by more and more advanced methodology, greatly enhanced knowledge about the biology of microorganisms. In the first half of the 20th century series of groundbreaking laboratory research work uncovered the basic components and processes of life, to a major degree using bacteria as objects of investigations (e.g. Avery, *et al*., 1944; Beadle and Tatum, 1941; Crick, *et al*., 1961; Franklin and Gosling, 1953; Jacob and Monod, 1961; Littlefield, *et al*., 1955; Meselson and Stahl, 1958; Watson and Crick, 1953a; Watson and Crick, 1953b; Wilkins, *et al*., 1953). Intriguing details about the bacterial structural organization, biochemistry, physiology, adaptation mechanisms, stress response, host-microbe, and microbe-microbe interactions have been revealed so far. About three decades ago it was re-discovered that in nature bacteria preferably live in multicellular communities, associated with terrestrial surfaces or animals and plants (e.g. Costerton *et al.*, 1987; Geesey *et al.*, 1977, Marshall, 1976; see also chapter 1.2). Since then, a major focus has been to unravel the genetic determinants and environmental conditions that promote and regulate the formation of these multicellular communities using so-called model organisms (e.g. Götz 2002; Klausen *et al.*, 2006; Lasa, 2006, see also chapter 2 and 3).

1.2 Bacterial lifestyles in nature: A binary view

In nature, no bacterium exists in isolation. Instead, each bacterial cell is part of a population of cells. Observations on bacteria revealed that they exist as populations of cells in which either i) the individual cells are spatially associated with each other and hence live in multicellular communities of a definable three-dimensional structure, termed the '*biofilm* mode of life', or in which ii) the individual cells are not firmly associated to each other, and hence are free living, termed the '*planktonic* mode of life' (Fig. 3). Although not mutually exclusive, biofilms are commonly found to be associated with solid and semi-solid matter, whereas planktonic cells are usually associated with liquid matter. Bacteria are capable of switching between the two modes of life and both modes of bacterial life are linked to other processes of life in nature. The following section will focus on the biofilm mode of life,

however, a brief outline about the role of the bacterial planktonic way of living will be given thereafter.

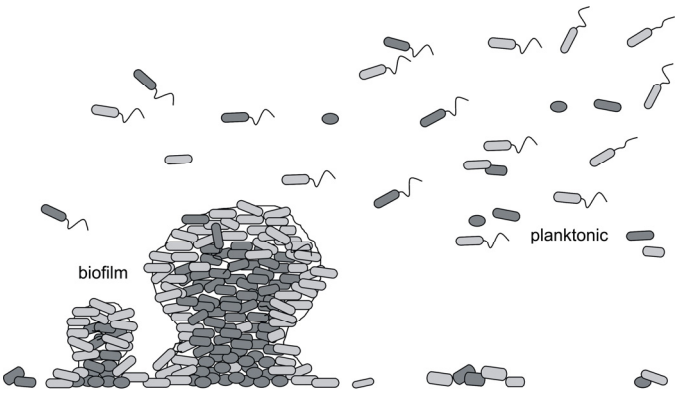

Fig. 3. Schematic illustration of the two bacterial modes of life. Populations of bacteria can be found in nature either as multicellular aggregates, so called biofilms (depicted to the left) or as free-living, so-called planktonic cells (depicted to the right). Observations indicate, that bacteria are able to switch between the two modes of life. Image is not drawn to scale.

1.2.1 The biofilm mode of life

Biofilms come in a great variety of shapes and sizes. Nevertheless, it appears that they share some common features. Biofilm cells are hold together by interconnecting compounds, such as excreted polysaccharides and proteins, cell lysis products and matter from the immediate surrounding environment, which altogether constitute the so-called 'matrix'. This allows cells to form long-term relationships and establish metabolic cooperations (e.g. Costerton *et al.*, 1987; Pamp *et al.*, 2007b).

Bacterial multicellular communities can be composed of mono-species populations of cells, such as the fruiting bodies formed by *Myxococcus* spp., or mycelial multicellular structures by *Streptomyces* spp. However, it appears that biofilms in nature predominantly are composed of cells derived from various species or even microorganisms of different domains (Davey and O'Toole, 2000). Stromatolites, as can be found in Western Australia (Fig. 4a) or the Bahamas, are recognized as ancient forms of biofilms (Reid *et al.*, 2000). Sedimentary rock formations in Pilbara Craton (Australia) have been found and, although controversial, claimed to be 3.4 billion years old fossils of stromatolites (Allwood *et al.*, 2006). Biofilms are frequently established on hydrous solid and semi-solid surfaces, such as soil, rock material, or surfaces of animals (Fig. 4b) and plants (Fig. 4c). However, biofilms at air/liquid interfaces can also be found, such as 'microbial mats' floating on the surface of waters (Fig. 4d) (Guerrero *et al.*, 2002; Steunou *et al.*, 2006). Water is essential for development and maintenance of biofilms, as it is generally critical for life, but also facilitates the transport and exchange of low molecular weight compounds, and dissemination of bacteria. The abundance of biofilms is many times higher in environments of high nutrient concentration; however, they can also form in nutrient-depleted environments, as well as in extreme habitats such as hypersaline, alkaline, or polar regions. Overall, the prevailing environmental conditions in a certain habitat greatly impact on the abundance and composition of biofilms (Costerton *et al.*, 1987; Davey and O'Toole, 2000; Marsh and Bowden, 2000).
Bacterial communities play key roles in food webs in nature as they perform degradation of detritus material and participate in nutrient cycling. Many of these processes are interdependent and require cooperation between various bacterial species with different metabolic capacities (Costerton *et al.*, 1987; Davey and O'Toole, 2000). The fact that in

biofilms the participating microbial members are situated in close proximity seems to be convenient, since metabolites can easily be transferred and metabolized further. Matrix components can support the adsorption of organic matter and other compounds and thereby provide a mechanism by which the community can concentrate nutrients. In cases of adverse conditions such as desiccation, osmotic shock, or exposure to toxic compounds, UV radiation, predators or other stresses, the microbial community as whole can provide protection. Moreover, multicellular communities provide ideal conditions for horizontal gene transfer, which is important for microbial evolution and genetic diversity (Costerton *et al*., 1987; Davey and O'Toole, 2000; Marsh and Bowden, 2000; Molin and Tolker-Nielsen, 2003).

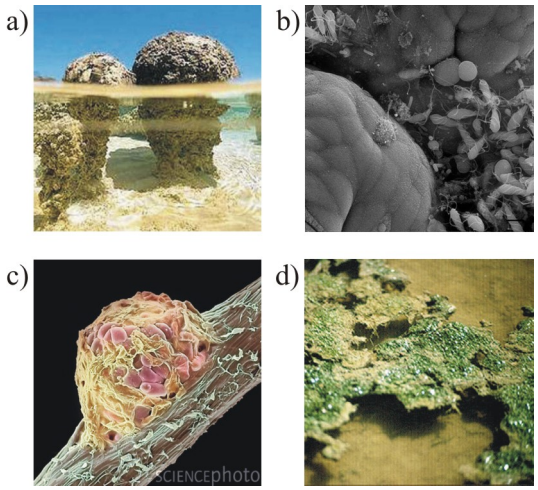

Fig. 4. The biofilm mode of life. *a)* Photograph of modern stromatolites (~3000 years old) from Shark Bay, Hamelin Pool Marine Nature Reserve, Western Australia (Source: www.ucl.ac.uk/star). Stromatolites are described to be formed by processes involving the development of mats of multispecies microbial communities (proteobacteria, planctomyces, actinobacteria, cyanobacteria), and binding of sedimentary grains (Burns *et al.*, 2004; Papineau *et al.*, 2005). *b)* Scanning electron micrograph (SEM) from the multispecies indigenous microbial population present in the mucous layer of the small intestine of a mouse (Source: Rozee *et al.*, 1982). *c)* Coloured scanning electron micrograph (SEM) of a root nodule on a pea plant (*Pisum sativum*) caused by the nitrogen-fixing bacteria *Rhizobium leguminosarum*. The bacteria convert atmospheric nitrogen to ammonia, and make it available to the plant. The plant passes carbohydrates to the bacteria as energy source (Credit: Jeremy Burgess/Science Photo Library). *d)* Photograph of a microbial mat floating on a roadside puddle (Credit: John Lennox, Penn State Altoona). Microbial mats are usually formed by multispecies microbial communities; among those cyanobacteria are common. Images are not shown to scale.

Biofilms as inhabitants of humans: impact on health and disease

It seems as intimate relationships between microbial communities and eukaryotic organisms are a dominant theme of life, as microbial communities frequently can be found living together with both animals (e.g. Fig. 4b) and plants (e.g. Fig. 4c). As known for humans, communities of bacteria natively populate mucous membranes and epithelial surfaces like the gastrointestinal tract, oral cavity, and skin. Each of the body sides is colonized with a mixed microbial community of characteristic composition (Wilson, 2005).

The human organism consist of approximately 10 times more prokaryotic cells than eukaryotic cells (Savage, 1977) and it has been estimated that alone the genomes of all intestinal microorganisms together contain roughly 100 times as many genes as does the entire human genome (Gill *et al.*, 2006). Intriguingly, for most of their lifespan humans do not suffer from harboring these microbial communities. In fact, they are important and

beneficial to their host as they can degrade nutrients and thereby make them accessible to humans and synthesize vitamins, which cannot be synthesizes by the human itself. Moreover, they play key roles in the development of the immune system and anatomy of the mucosal surfaces and exert protective function against exogenous pathogens (Berg, 1996; Noverr and Huffnagel, 2005; Wilson, 2005).

The relationship between the host and its microbial communities is delicately balanced but under certain conditions it can break down and result in infectious diseases. These infections can be caused either by members of the indigenous human microbial community or by microorganisms from the environment (Costerton *et al.*, 1987; Wilson, 2005). Under conditions where the host is impaired, for example immunocompromised, injured, or suffering from cancer or cystic fibrosis, harmful biofilms can develop at different body sides and cause persistent infections. Among those infections are various device-related infections, but also otitis media, osteomyelitis, pulmonary infections, infective endocarditis, wound infections, and chronic prostatitis (Costerton *et al.*, 2003; Hall-Stoodley *et al.*, 2004; Parsek and Singh, 2003). The most common infections caused by microorganisms of the human indigenous microbial community are caries, periodontal diseases and urinary tract infections (Wilson, 2005). Bacteria which in general have been found to be involved in human biofilm-related infections are Staphylococci, *Escherichia coli*, *Pseudomonas aeruginosa*, Enterococci, Streptococci, *Proteus mirabilis*, *Klebsiella*, *Enterobacter*, and *Haemophilus influenza*. Some of these persistent infections involve either of these opportunistic pathogens, however, it appears that many are polymicrobial infections, since various species can be identified from the side of infection (Costerton *et al.*, 2003; Hall-Stoodley *et al.*, 2004). The significance of single species in a particular persistent infection is often unknown.

A general characteristic of persistent infections is, that they evidently cannot be eradicated by the host immune system and are difficult to kill by antimicrobial chemotherapy. Various reasons have been postulated to be involved in the persistence of these infections: i) biofilm matrix compounds may protect the bacteria, ii) bacteria living in biofilms exhibit a biofilm-specific antibiotic-resistant physiological state, iii) the causative bacteria have a decreased growth rate, iv) the biofilm is composed of a heterogeneous population of cells exhibiting different physiological states, which renders only some cells vulnerable to attack by antimicrobial compounds, v) biofilm-growth *per se* increases induction of bacterial stress response, which protects the cells against antimicrobials, and vi) quorum sensing systems might confer protective functions (Fux *et al.* , 2005; Lewis, 2001; Mah and O'Toole, 2001; Stewart, 2002).

1.2.2 The planktonic mode of life

It seems that only a minor fraction of all bacteria on Earth are living in the planktonic mode, since most bacteria are found to be associated with terrestrial habitats (incl. oceanic subsurfaces) compared to the aquatic habitats, such as the global oceans, lakes and rivers (Whitmann *et al.*, 1998). Nevertheless, this mode of life can play important roles in bacterial ecology. Whereas some species might be found predominantly in the planktonic mode of growth, others exist in both, the planktonic and biofilm mode of life.
In the global oceans, bacterioplankton lives almost exclusively in the planktonic form. The cyanobacteria *Prochlorococcus* (Fig. 5a) and *Synechococcus* (Fig. 5b) belong to the most abundant bacteria found in the oceans and account for a significant amount of photosynthesis carried out in nature. Differences in the composition of their photosynthetic light harvesting pigment complexes, and resulting distinct absorptions spectra, but also nutrient requirements appear to play role in differentiating their ecological niches in the

ocean environment: Whereas *Synechococcus* seems to be prevalent in almost all areas, *Prochlorococcus* is restricted to the tropical and subtropical regions. In open waters, where both species occur, *Prochlorococcus* has been found to extend deeper in the water column than *Synechococcus* (Partenski *et al.* 1999; Ting *et al*., 2002).

Another interesting group of preferentially planktonic living bacteria seem to be magnetotactic bacteria. Magnetotactic bacteria are a morphologically and taxonomically diverse group of motile aquatic microorganisms (e.g. Fig. 5c). They are found in high numbers at the oxic-anoxic transition zone within calm marine and freshwater environments. Research indicates, that they organize themselves by flagella-driven motility at this particular aquatic zone steered by magnetotaxis and aerotaxis. Magnetotaxis is carried out using intracellular, self-synthesized crystals of magnetic minerals, so-called magnetosomes. It is assumed that these organisms play role in iron, sulfur and carbon cycling (Blakemore, 1982; Bazylinski and Frankel, 2004).

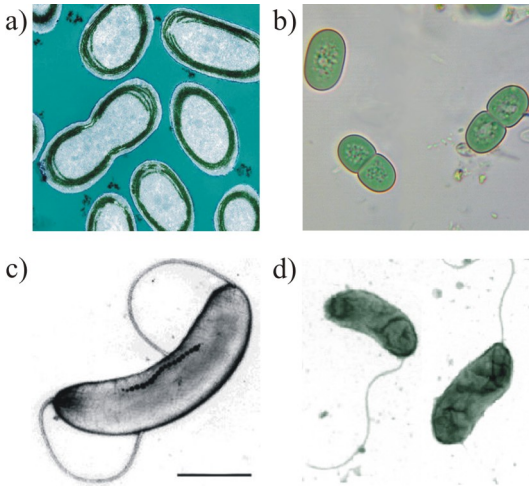

Fig. 5. The planktonic mode of life. *a)* Colored transmission electron micrograph (TEM) of a section through *Prochlorococcus* cells. The section reveals bands of membranes (green) inside the cells, which contain the light harvesting pigment complexes, used for photosynthesis (Credit: Claire Ting/Science Photo Library). *b)* Photomicrograph of *Synechococcus* cells (Credit: Ralf Wagner, Germany). Both *Synechococcus* and *Prochlorococcus* are thought to play important roles in regulating carbon dioxide and oxygen levels in the atmosphere. *c)* Transmission electron micrograph (TEM) of a *Magnetospirillum magnetotacticum* cell. The magnetosomes can be seen within the cell as a row of dark square-like objects (Credit: R. B. Blakemore and N. Blakemore). *d)* Transmission electron micrograph (TEM) of *Vibrio cholerae*. (Credit: New York State Department of Health, Wadsworth Center). Images are not shown to scale.

Generally, the bacterial planktonic way of living provides the opportunity to rapidly change location and penetrate into new ecological niches. This seems to be an attractive strategy in cases of nutrient limitation or other external adverse conditions in a particular environment. Moreover, the planktonic mode can facilitate the dissemination of bacteria from a biofilm community, for example in aging populations or when adverse conditions occur. In relation to human infectious diseases it appears that both, the planktonic as well as the biofilm mode of growth can be integral parts of pathogenesis. An interesting case is described for *Vibrio cholerae* (Fig. 5d). This organism exists in the environment predominantly in the biofilm mode attached to zooplankton. Upon oral digestion by humans, cells detach from biofilms in the intestine to become planktonic. Subsequently, cells colonize the intestinal epithelium, multiply and form biofilm-like aggregates. In later stages of this infectious process large numbers of bacteria emigrate from the epithelial surface, mainly planktonically but also as

aggregates, to leave the human environment and enter possibly again aquatic environments (Colwell, 2004; Faruque *et al.*, 2006; Nielsen *et al.*, 2006; Zhu and Mekalanos, 2003). Another example for dissemination of planktonic bacteria during infectious diseases can be observed in cases of bacteremia. In these cases bacteria can be found emigrating from biofilms, which had formed on indwelling devices, such as catheters. Bacteremia can lead to severe sepsis and multiple organ failure (Eggimann *et al.*, 2004).

1.3 Techniques for cultivation and analysis of biofilms

Biofilms are intriguing societies of microbes and a great mission is to unravel their structural assembly, organization, physiology, evolution and role in nature. However, due to their complexity, natural microbial communities have been challenging objects of investigation. Various factors impact on biofilm biology and many of those factors are interrelated. To reduce complexity and facilitate investigations in the laboratory under controlled and reproducible conditions, various 'simple' methods for biofilm cultivation and examination have been established. Moreover, to discover genetic determinants and regulatory pathways impacting on the biofilm mode of life, major focus relies on well characterized and genetic tractable microorganisms, such as *Pseudomonas* spp., Staphylococci, *Bacillus subtilis*, *E. coli, V. cholera*, *Salmonella typhimurium* and *Candida albicans*. Generally, research on laboratory-grown biofilms applies combinatorial approaches, which can involve different methods of biofilm cultivation, macroscopic examinations, microscopic examinations, quantifications, computational modeling, genetic engineering and functional genomics. The following section should give an overview about the most common tools used to study biofilm biology of these model organisms.

1.3.1 Flow-chamber-grown biofilms

Flow-cell technology in combination with confocal laser scanning microscopy (CLSM) and fluorescent reporters appears to be the gold standard in biofilm research. It allows getting insight into details of developmental processes, spatial organization and function of biofilms under continuous and non-invasive conditions up to the single-cell level (e.g. Palmer *et al.*, 2006). In flow-chambers, biofilms are cultivated under hydrodynamic conditions and parameters such as nutrient composition, flow-rate, temperature and oxygen supply can be chosen as required (schematic setup of the system: Fig. 6a). As surface for microbial attachment serves glass, however, the surface may be modified by coating. CLSM allows the visualization of fully hydrated biofilms on-line in up to four dimensions. To be able to detect living cells present in the biofilm via CLSM, the cells need to either express a 'fluorescent tag' such as the green, cyan, yellow, or red fluorescent protein (Gfp, Cfp, Yfp or Rfp) (e.g. Fig. 7a), or be stained with a fluorescent dye (e.g. Syto 9 or Syto 62). Moreover, visualization of dead cells is possible using e.g. propidium iodide, and matrix components can be visualized using fluorescent dyes (e.g. calcofluor white) or fluorescently labeled lectins (e.g. tetramethylrhodamine-wheat germ agglutinin (TMR-WGA)) (Doyle, 1999; Palmer *et al.*, 2006). To assess the role of particular genes in biofilm formation, knock-out mutants can be genetically engineered and the phenotype of these strains compared with the phenotype of the isogenic wild type strain (e.g. Fig. 7a). The expression of single genes of interest can be studied *in situ* using fluorescent promotor fusions. In this case a gene encoding for a fluorescent protein (e.g. *gfp*, *gfp*[AGA]) is placed under expression control of the promotor of the gene of interest, and this construct introduced into the microbial cell. Only cells in which the expression from the promotor of interest is induced will exhibit a fluorescent signal (Andersen, *et al.*, 1998). Fluorescent *in situ* hybridization (FISH) can be applied to target specific nucleic acid sequences of biofilm

cells, however, this requires fixation and embedding procedures and removal of the biofilm from the flow-chambers. Depending on the equipment of the CLSM (lasers, filter sets) it is possible to combine some of the visualization techniques described above (Doyle, 1999; Palmer *et al.*, 2006). Certain general features of biofilms can be quantified, such as biofilm thickness and roughness, biovolume and substratum coverage (Heydorn *et al.*, 2000). Furthermore, the response of biofilms to changes in environmental conditions can be studied by exposing the biofilm to e.g. another carbon source, high shear force, antimicrobial compounds, or predatory eukaryotic cells (e.g. Palmer *et al.*, 2006).

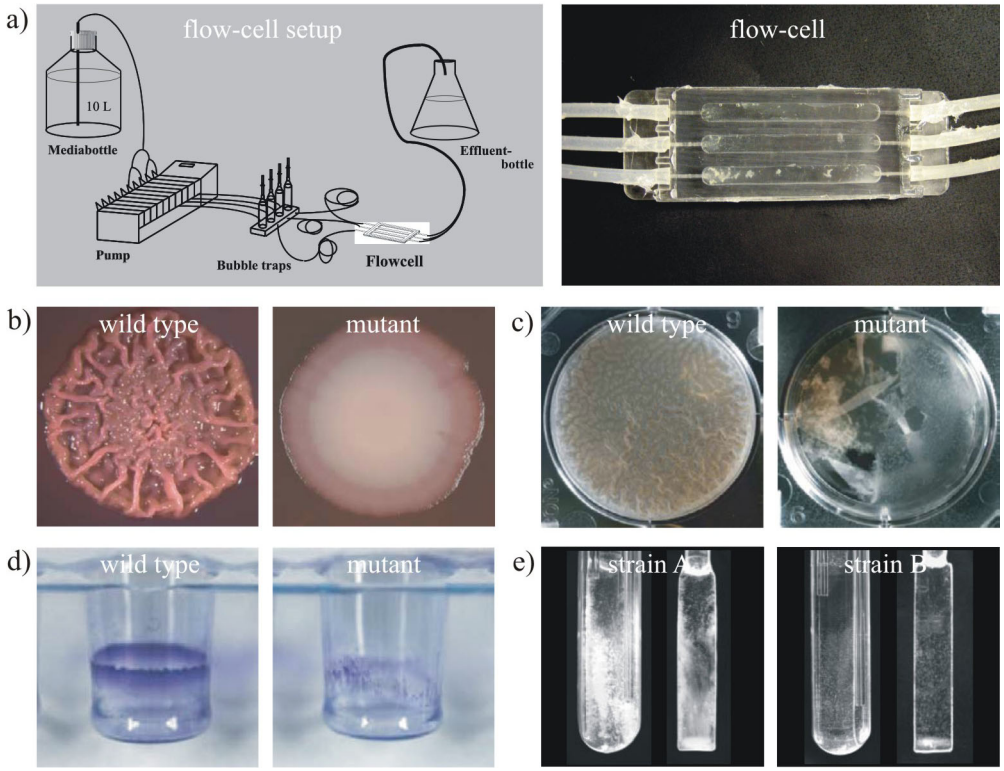

Fig. 6. Cultivation of biofilms. *a)* To the left: Schematic representation of the flow-cell setup (Credit: K. B. Barken and J. A. J. Haagensen). Biofilms are cultivated in individual flow-channels of three-channel-flow-cells (to the right) (Image: Pamp, unpublished). A peristaltic pump is used to attain a continuous flow of media through the flow-chambers and bubble traps are interconnected to catch air bubbles, which can accidentally arise in the system. *b)* Colony biofilms of *P. aeruginosa* strains on agar plates containing Congo red dye. The morphology of the wild type (to the left) appears wrinkly due to production of matrix components. The mutant strain (to the right) is defective in matrix synthesis (Source: Branda *et al.*, 2005). *c)* Pellicle biofilms of *B. subtilis* strains formed at the air/liquid interface of standing cultures. The mutant strain (to the right) is defective in matrix production and unable to form a stable pellicle, as formed by the wild type strain (to the left) (Source: Branda *et al.*, 2005). *d)* Biofilms formed in the wells of a microtiter dish by *E. coli* wild type (to the left) and a mutant strain of *E. coli* (to the right). Biofilms are visualized by staining with crystal violet (Source: Branda *et al.*, 2005). *e)* Biofilms formed by *E. coli* strains on Pyrex glass slides in microfermentors. Strain A harbors two plasmids encoding for conjugative type IV pili, whereas strain B does not contain any plasmids (Source: Dudley *et al.*, 2006). Images are not shown to scale.

1.3.2 Other experimental biofilm setups and tools

The bacterial colony itself has been recognized as a type of biofilm and proven to be a useful object for biofilm investigations. Cultivations of colony biofilms do not require any specific and costly equipment and they are applicable for screenings, such as transposon-libraries. Bacteria are allowed to establish a colony biofilm either directly on the surface of an agar-plate (Fig. 6b), or on top of a polycarbonate membrane, which is placed on an agar-

plate (Bokranz *et al.*, 2005; Branda *et al.*, 2004; Werner *et al.*, 2004). The morphology of these colonies can vary greatly, dependent on the bacterial species used and the intrinsic ability of a particular strain to produce matrix-compounds, such as polysaccharides and proteins (Fig. 6b) (Bokranz *et al.*, 2005; Branda *et al.*, 2004). Moreover, environmental conditions such as the prevailing media composition, agar concentration, temperature, and duration of cultivation can impact on the morphology of the colony biofilm. The presence of matrix components can be examined by applying dyes, which specifically bind to these components (e.g. Congo red) (Fig. 6b) (Bokranz *et al.*, 2005). Microscopy such as scanning electron microscopy (SEM) or transmission electron microscopy (TEM), have also been helpful in visualizing cell-to-cell interconnecting components (e.g. Fig. 7b+c). Studying dynamics and developmental processes within the colony is difficult as it is not possible so far to follow these processes under non-destructive conditions. However, *in situ* gene expression studies are achievable in combination with embedding and cryosectioning procedures and subsequent microscopic investigations (Fig. 7d) (Werner *et al.*, 2004). Functional genomics approaches, such as transcriptomics (DNA-microarrays) or proteomics (e.g. 2D-PAGE) may be applied on these biofilms if desired (e.g. Mikkelsen *et al.*, 2007; Waite *et al.*, 2005).

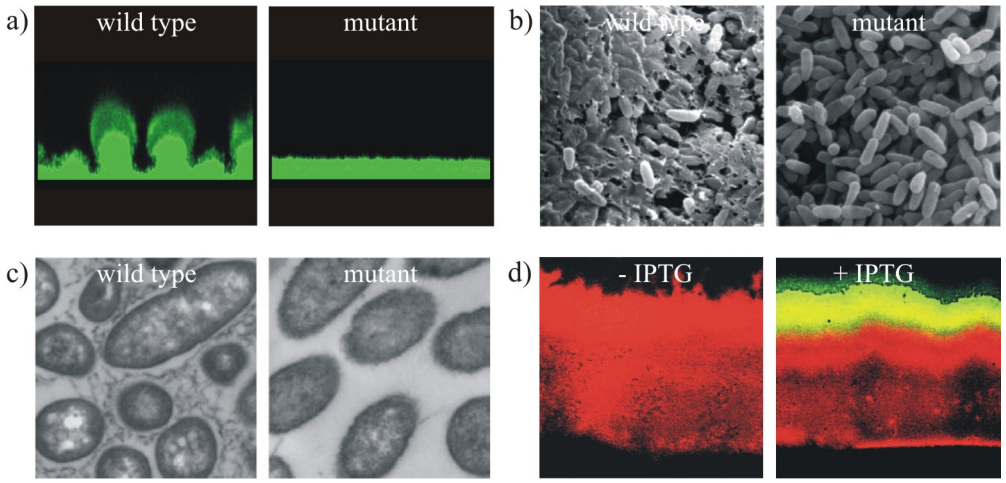

Fig. 7. Microscopic examination of biofilms. *a)* Confocal laser scanning micrographs (CLSM) of flow-chamber grown *P. aeruginosa* strains (vertical representation). *P. aeruginosa* PAO1 wild type (Gfp-tagged) forms a mushroom-like structured biofilm, whereas *P. aeruginosa* PAO1 *rhlA*::Gm^r (Yfp-tagged) forms a flat-structured biofilm (Images: Pamp, unpublished). *b)* Scanning electron micrograph (SEM) of *P. aeruginosa* biofilms. The *P. aeruginosa* wild type strain produces matrix-compounds, whereas the mutant strain is defective in matrix-synthesis (Source: Branda *et al.*, 2005). *c)* Transmission electron micrograph (TEM) of *P. aeruginosa* biofilms. The *P. aeruginosa* wild type produces matrix-compounds, visualized with ruthenium red, whereas the mutant strain is defective in matrix-synthesis (Source: Branda *et al.*, 2005). *d)* Confocal laser scanning micrographs (CLSM) of vertical cryosections of colony biofilms formed by *P. aeruginosa*, visualized with rhodamine B. The *P. aeruginosa* strain harbors a *gfp*-gene, which expression is inducible by IPTG. Gfp is not expressed in the absence of IPTG (to the left). In the presence of IPTG (to the right), Gfp is expressed in the upper layer of the colony biofilm in metabolically active cells (Source: Werner *et al.*, 2004). Images are not shown to scale.

Biofilms can also form at air/liquid interfaces. In this regard, so-called pellicle biofilms can be established at the air/liquid interface of static cultures. It appears that matrix-production is explicitly required for establishment of a pellicle. Strains that are defective in matrix-production do not form a pellicle at the air/liquid interface or only a fragile layer of cells (Fig. 6c) (e.g. Branda *et al.*, 2004). Environmental conditions, such as media composition and temperature can effect on pellicle formation. Cell-to-cell interconnecting components can be visualized by either scanning electron microscopy (SEM) or transmission electron

microscopy (TEM) (e.g. Fig. 7b+c) (e.g. Anriany *et al.*, 2001). This biofilm assay is also compatible with high-throughput screenings. For detailed microscopic analysis, aimed at learning about structural organization and in *situ gene* expression, this assay is not well suitable.

The microtiter-dish assay is a popular biofilm assay in which also the amount of biofilm can be easily quantified. Moreover it can be applied in large scale, such as for screenings of transposon-libraries. Commonly cultures with a low inoculum of bacteria are introduced into the wells of the microtiter-dish and incubated under static condition. Biofilms can form at the walls of the wells and subsequent to washing-steps stained with dyes such as crystal violet. The amount of crystal violet bound is assumed to be directly proportional to the amount of biofilm, and therefore the amount of biofilm is determined by extraction of the crystal violet from the biofilm and subsequent quantitative absorptions measurements (e.g. O'Toole and Kolter, 1998a). The effect of environmental conditions on biofilm formation can be easily investigated, e.g. by modifying the composition of the media, addition of compounds of interest, variation of the incubation temperature or duration. However, this assay is not suitable for microscopic investigations concerning biofilm structural development.

Biofilms can also be cultivated in microfermentors. Here the biofilm can develop on Pyrex glass slides under continuous flow condition and the flow rate can be adapted to minimize the amount of planktonic cells. The biofilm biomass can be recovered and quantified, for example by measurements of optical densities of resuspensions of biofilm cells. The structure of the biofilm can be investigated by microscopy, such as scanning electron microscopy (SEM) (e.g. Fig. 7b). Global approaches, like transcriptomics (DNA-microarrays) or proteomics (e.g. 2D-PAGE) can be applied on these biofilms as well. Moreover this setup is applicable for small-scale genetic screenings. The role of environmental conditions can be investigated, e.g. by modifying the media composition, incubation temperature or addition of antimicrobial compounds (Ghigo, 2001; Dudley *et al.*, 2006; Valle *et al.*, 2006).

2 Cellular differentiation in single species bacterial multicellular communities

This chapter aims at providing insight into the organization of multicellular communities at the microscopic level. Using examples from various laboratory-grown single species multicellular communities, a number of different cell types will be introduced which can evolve by cellular differentiation. The phenotypes of the different cell types will be described as well as their impact on the development of a multicellular community. Thereafter, additional features of some cell types in relation to biofilm cell populations, and cell types in relation to planktonic cell populations, will be discussed.

2.1 Cell types in multicellular communities

The biofilm mode of life is described to be distinct from the planktonic mode of life. Comparative analysis of the global transcriptional profiles of planktonic cell populations and biofilm cell populations demonstrated that there exist significant differences between these two populations (e.g. Beloin *et al.*, 2004; Resch *et al.*, 2005; Whiteley *et al.*, 2001). Also at the global proteomic level populations of planktonic cells differ significantly from populations of biofilm cells (e.g. Mikkelsen *et al.*, 2007; Resch *et al.*, 2006; Sauer *et al.*, 2002). Together this suggests, that the physiology of biofilm cells significantly differs from the physiology of planktonic cells.

Investigations at a lower level of organization reveal that single species multicellular communities by a number of bacteria, such as *P. aeruginosa*, *B. subtilis*, *Myxococcus xanthus*, *Streptomyces* spp., and Staphylococci, consist of a variety of different cell types exhibiting various physiological states and distinct phenotypes. Among those cell types can be e.g. metabolic active cells, metabolic inactive cells, motile cells, non-motile cells, matrix-producing cells, dead cells, spore cells, and genetic variant cells. Some of these cell types are mutually exclusive, e.g. spore cells are generally not motile. However, cells might also exhibit overlapping phenotypes, e.g. metabolic active cells can be motile. It appears that cells of the same type are often found to be spatially co-localized forming confined subpopulations within the multicellular community. The following section aims at introducing some of the various cell types, which have been found to be present in a number of laboratory-grown multicellular communities.

High metabolic active cells / low metabolic active cells

Microscopic analysis on various laboratory-grown biofilms, e.g. by *P. aeruginosa*, *S. epidermidis*, *Klebsiella pneumoniae, E. coli* and *P. putida,* reveal that biofilm cells exhibit differential degrees of metabolic/physiological activity. The biofilm cells appear to fall into two major spatially distinct subpopulations: A subpopulation of cells situated proximal to the substratum exhibits low metabolic/physiological activity, whereas a subpopulation of cells distal to the substratum at the biofilm/air or biofilm/liquid interface exhibits high metabolic/physiological activity. The spatial distribution of low and high metabolic/

physiological active cells appear to be a result of prevailing low and high local concentrations of oxygen, respectively, however, also local limitations of nutrient compounds might result in a lower metabolic activity of cells situated in the deeper layers of the multicellular structure (Pamp *et al.*, submitted; Rani *et al.*, 2007; Sternberg *et al.*, 1999; Wentland *et al.*, 1996; Werner *et al.*, 2004; Haagensen, personal communication).

Motile cells / non-motile cells

A binary cellular phenotype with respect to cellular migration can be observed in a number of multicellular communities, e.g. by *P. aeruginosa*, *M. xanthus*, and *B. subtilis*, as both, motile and non-motile cells can be found to co-exist in the respective multicellular structures. In the initial phase of flow-chamber-grown biofilms of *P. aeruginosa* the substratum-attached cell population appears to differentiate into a motile (driven by type IV pili) and non-motile fraction of cells (Klausen *et al.*, 2003b; Singh *et al.*, 2002; see also chapter 3.2). During further developmental steps spatially confined microcolonies are formed by clonal proliferation of non-motile cells. A fraction of motile cells can migrate on top of the microcolonies to initiate the formation of a spatially confined cap-forming subpopulation. Migration of these cells seems to be facilitated by flagella-driven motility and the secretion of biosurfactants and depends on the presence of type IV pili and extracellular DNA. In aged multicellular communities of *P. aeruginosa* rapidly swimming cells can be observed inside hollows formed in the center of the structures. These cells appear to function as offspring as they can leave from the multicellular structures and possibly establish in new niches (Barken *et al.*, in prep.; Haagensen *et al.*, 2007; Klausen *et al.*, 2003b; Pamp and Tolker-Nielsen, 2007; see also chapter 3.2). Motile and non-motile cells appear also to be involved in the formation of multicellular communities (fruiting bodies) by *M. xanthus*. Cells can move along surfaces by means of so-called gliding motility, which is driven cooperatively by S-motility (type IV pili, extracellular fibril material, LPS) and A-motility (slime secretion). In cases where groups of gliding cells collide, small irregular cellular aggregates (‘traffic jams’) are formed and the participating cells become non-motile (Fig. 8a). When streams of other gliding cells encounter such an aggregate it can serve as a nucleus for enlargement: the stream glides over and around the aggregate to produce a hemispherical mound on top of the subpopulation of non-motile cells. Here the cells move in cyclic paths dependent on cell-to-cell contact facilitated by the C-signal (surface-bound protein). As the mound increases in size motile cells can differentiate into non-motile spores (Kaiser, 2003; Sliusarenko *et al.*, 2007; Sozinova *et al.*, 2006). A distinct subpopulation of motile cells can also be observed in colony biofilms by *B. subtilis* (Fig. 8b). In contrast to the described multicellular communities by *P. aeruginosa* and *M. xanthus*, the motile cells (driven by flagella) in the *B. subilis* biofilm are predominantly found at the base. It is hypothesized that motile cells might actively move to the base attracted by nutrients present in the agar medium. Nevertheless, some motile cells were also observed in the top layer of the biofilm (Kolter, 2007; Vlamakis *et al.*, 2007).

Matrix-producing cells

Matrix synthesis appears to be spatially confined to distinct subpopulations of cells in laboratory-cultivated multicellular communities such as *P. aeruginosa*, *M. xanthus*, and *B. subtilis*. In *P. aeruginosa* flow-chamber-grown biofilms, the polysaccharide PEL seems to be preferably produced by cells, which are located in the upper layer of microcolonies and mushroom-like structures. Extracellular DNA seems to be localized preferably inside and in the upper part of microcolonies and at the interface between the stalk-forming and cap-forming subpopulations of mushroom-like structured biofilms. In early stages of biofilm formation the extracellular DNA seems to play role in stabilization of initial microcolonies. In subsequent stages the extracellular DNA is important for development of the cap-

forming subpopulation lifting up from the stalk-forming subpopulation (Allesen-Holm *et al.*, 2006; Ma *et al.*, 2007b; Whitchurch *et al.*, 2002). *M. xanthus* cells produce an extracellular fibril matrix, composed of polysaccharide and protein, which is thought to surround the single cells. A recent study reports that the polysaccharide component can induce retraction of type IV pili and thereby modulate S-motility. In mature multicellular structures the polysaccharide component has been found to be present in the outer layers of the fruiting bodies, suggesting that cells in the outermost layers exhibit increased polysaccharide synthesis (Behmlander and Dworkin, 1994; Li *et al.*, 2003; Lux *et al.*, 2004). *B. subtilis* produces a matrix, which is composed of a polysaccharide component (EPS) and a protein component (TasA). Recent investigations indicate that in colony biofilms by this organism, TasA expression is not uniform throughout the biofilm, instead it is confined to certain cell-groups, which are scattered throughout the multicellular structure (Aguilar *et al.*, 2007; Kolter, 2007).

Dead cells

Dead cells and remnants of cells (e.g. extracellular DNA, and cell envelopes devoid of cytoplasmic contents) can be found in a number of multicellular communities, such as by *P. aeruginosa*, Staphylococci, and *Streptomyces* spp. In biofilms of *P. aeruginosa* some dead cells can be found in the stalk-part of the multicellular structure close to the substratum. Extracellular DNA seems to be derived from lyzed cells, and can be found in microcolonies and as layers between the stalk- and cap-forming subpopulations (see above, matrix-producing cells). The number of dead cells and amount of extracellular DNA can vary dependent on environmental conditions, age of the biofilm and inherent characteristics of a particular strain. Factors such as anoxic microenvironments, reactive nitrogen and oxygen intermediates, phage induction, flagella, type IV pili, and quorum sensing have been implicated with the appearance of dead cells and extracellular DNA in *P. aeruginosa* biofilms. (Allesen-Holm *et al.*, 2006; Barraud *et al.*, 2006; Hunt *et al.*, 2004; Hunter and Beveridge, 2005; Webb *et al.*, 2004; Werner *et al.*, 2004; Whitchurch *et al.*, 2002). In biofilms by Staphylococci dead cells and extracellular DNA were also observed in the interior area of the multicellular structures. In *S. aureus* cell lysis is suggested to be dependent on the *cid* and *lrg* genes, which are homologous to genes that encode for proteins mediating murein hydrolase-dependent cell lysis. In *S. epidermidis* the appearance of dead cells and extracellular DNA in biofilms appears to be dependent on autolysin (AtlE) (Qin *et al.*, 2007; Rice *et al.*, 2007). Dead cells have also been found to be part of mycelial colonies by *Streptomyces* spp. and appear to be important for structural development of these colonies. Generally, the mycelial colonies consist of two major cell types: the vegetative substrate mycelium present in and on the substratum, and aerial hyphae arising from the substratum into the air (Fig. 8c). Development of aerial hyphae is described to be dependent on cell death of the substrate mycelium. In early stages of colony development the substrate mycelium is formed. In response to nutrient starvation specialized aerial hyphae can arise, which grow upwards into the air. Growth of aerial hyphae appears to coincide with cell death of the substrate mycelium. It is thought that remnants of these dead cells might serve as nutrient supply for aerial hyphae development. Some hyphae die by autolyses, however, the majority of hyphae undergo a process termed physiological cell death. This is a slower process in which cellular contents such as DNA degrade, whereas the cell envelope maintains shape for a while. Eventually the cell contents of dead cells are released leaving an empty cell envelope. This is proposed to be advantageous, as remains of cell envelopes might provide some mechanical support for aerial hyphae development. Death of the substrate mycelium coincides with the release of antimicrobial substances and it is thought that this is a mechanism by which possible other invading microorganisms (attracted by the

release of nutrients from dying hyphae) are kept away (Chater, 1993; Claessen *et al.*, 2006; Miguélez *et al.*, 2000).

Surfactant-producing cells

Release of surface active agents appears to be confined to distinct areas within multicellular communities and facilitates differentiation of distinct cell subpopulations in laboratory-cultivated multicellular communities such as *P. aeruginosa*, *Streptomyces* spp., and *B. subtilis*. In *P. aeruginosa* flow-chamber-grown biofilms, excretion of biosurfactants seems to have various effects. They are preferably produced by cells, which form microcolonies during early stages of biofilm formation, and by cells, which constitute the stalk of mushroom-like structures during later stages of biofilm formation, suggesting that they exert effects during different stages of biofilm development. Consistent with this, a *rhlA*-mutant was found to be affected in microcolony formation, possibly because biosurfactants are required for cell-to-cell-aggregation in early stages during microcolony formation. During later stages biosurfactants appear to be involved in formation of the cap-forming subpopulation by facilitating migration of motile cells onto the microcolonies, and by keeping the water channels open in between the mushroom-shaped structures. In aging biofilms biosurfactants are reported to facilitate the detachment of cells from the multicellular structures (Boles *et al.*, 2005; Davey *et al.*, 2003; Lequette and Greenberg, 2005; Pamp and Tolker-Nielsen, 2007). Surfactant components have also been found to be involved in structural development of mycelial colonies by *Streptomyces* spp, by facilitating the differentiation of aerial hyphae from the vegetative substrate mycelium. It appears that the surface tension between the aqueous milieu of the colony and the air must be reduced if nascent hyphae are to grow into the air. Dependent on the composition of the growth medium, this can be accomplished by two classes of surfactant-like molecules: the SapB peptide and the chaplin proteins. SapB is a lanthionine-containing peptide, which is thought to fold into a three-dimensional structure exhibiting amphiphilic characteristics. Observations suggest that SapB is expressed in the substrate mycelium and aerial hyphae, but not in spore cells. The chaplins form proteinaceous fibrils that mediate the rigidity of aerial hyphae, keep aerial hyphae apart and provide surface hydrophobicity. Expression of chaplins is confined to aerial hyphae and spores, however, in contrast to aerial hyphae formation chaplins are not required for the formation of spores (Capstick *et al.*, 2007; Claessen *et al.*, 2003; Claessen *et al.*, 2006; Elliot *et al.*, 2003; Kodani *et al.*, 2004; O'Connor *et al.*, 2002; Tillotson *et al*., 1998; Willey *et al.*, 2006). Aerial structures, which are reminiscent from fruiting bodies of other microorganisms, can also arise from the upper layers of colony and pellicel biofilms by *B. subtilis* (Fig. 8b). Surfactin, a biosurfactant produced by *B. subtilis*, appears to be involved in the formation of the aerial structures. Surfactin is thought to either facilitate erection of the aerial structures by lowering surface tension, or mediate cell-to-cell signaling and thereby modulate the expression of other factors required for structural development of the multicellular community and the aerial structures (e.g. TasA) (Branda *et al.*, 2001; Kolter, 2007)

Endospore cells

Differentiation of spore cells appears to take place in distinct areas of multicellular communities by a number of microorganisms, e.g. *M. xanthus*, *B. subtilis*, and *Streptomyces* spp. As described above, *M. xanthus* can undergo a developmental cycle to form fruiting bodies (Fig. 8a). In the hemispheric multicellular part, which is lifted up from the substratum, cells can differentiate into spore cells. When the C-signal has reached a certain threshold, rod-shaped motile cells differentiate into round non-motile spores accumulating in the center of the fruiting body. It is thought that spore cells are formed in the outer layer of the fruiting body and then passively transported due to encountering moving cells

(present in the outer layer) into the center of the multicellular structure (Kaiser, 2003; Sliusarenko *et al.*, 2007; Sozinova *et al.*, 2006). Differentiation of spore cells appears to take place also in distinct parts of multicellular colony and pellicle structures by *B. subtilis* (Fig. 8b). It has been observed, that sporulation-specific gene expression is confined to the top part of the aerial structures erecting from colonies and pellicles, indicating that these are the predominant sites of spore formation. Transcriptional regulators, such as Spo0A, sigma H and sigma F are found to be required for structural development of the overall multicellular community and/or differentiation of spores (Branda *et al.*, 2001). Spores can also develop at the top of aerial hyphae of mycelial colonies by *Streptomyces* spp. (Fig. 8c). Upon a complex differentiation process the extension of aerial hyphae stops and hyphal-tips undergo multiple septation. This gives rise to compartments in which spores develop. The process appears to be dependent on a second round of cell death (see information regarding the first round of cell death above), which here affects the basal non-sporulating parts of the aerial hyphae (Chater, 1993; Claessen *et al.*, 2006; Miguélez *et al.*, 2000).

Genetic variant cells

When biofilm cells are harvested and plated onto nutrient agar a diversity of colony morphology variants can appear. Biofilm-derived colony variants that differ in morphology from their respective wild type ancestor have been described for bacterial species such as *P. fluorescence*, *P. aeruginosa*, *Listeria monocytogenes*, *Serratia marcescens*, *S. pneumoniae*, and *S. aureus* (Allegrucci and Sauer, 2007; Boles *et al.*, 2004; Kirisits *et al.*, 2005; Koh *et al.*, 2007; Monk *et al.*, 2004; Rainey and Travisano, 1998; Yarwood *et al.*, 2007). As the phenotype of these variants in many cases is reported to be stable, it is assumed that genetic variations (e.g. mutations) in some biofilm cells occurred resulting in the observed altered colony phenotypes. However, a direct causal relationship between a genetic variation and an altered phenotype is documented in the rare case. At present it is unknown if the genetic variant cells are generated and localized in a certain spatial area within the biofilm structure, or whether these cells are randomly distributed throughout the multicellular structure. A number of different colony variants of *P. aeruginosa* have recently been reported to arise at high frequency in biofilms cultivated in a drip-flow biofilm reactor (Boles *et al.*, 2004). Among the most prominent variants were found ‘wrinkly’ and ‘mini’ colony morphotypes. Beside their altered colony morphology the various colony variants exhibited differential degrees of swimming motility, auxotrophy, pigment production and two isolated wrinkly and mini variants exhibiting altered biofilm formation phenotypes in flow-chambers. Further analysis indicate that a *recA*-dependent process is involved in generating the diversity of colony variants, as plated biofilm cells by a *recA*-mutant exhibit only wild type colony morphology (Boles *et al.*, 2004). Recent investigations point at the involvement of oxidative stress in the generation of genetic variants. It is hypothesized that reactive oxygen intermediates prevailing in these biofilms might induce double strand DNA breaks and that a mutagenic repair might result in the generation of various genetic variants (Singh, 2007). In another study a ‘sticky’ colony morphotype was isolated from *P. aeruginosa* biofilms established in a tube reactor (Kirisits *et al.*, 2005). This variant exhibited a strong auto-aggregative phenotype, hyperadherence to abiotic surfaces and lower degrees of swimming and twitching motility. In flow-chambers the variant formed biofilms with increased biomass compared to the wild type ancestor. Further investigations revealed, that the auto-aggregative and hyperadherence phenotypes were partly mediated by an increased production of the polysaccharide PSL (Kirisits *et al.*, 2005).

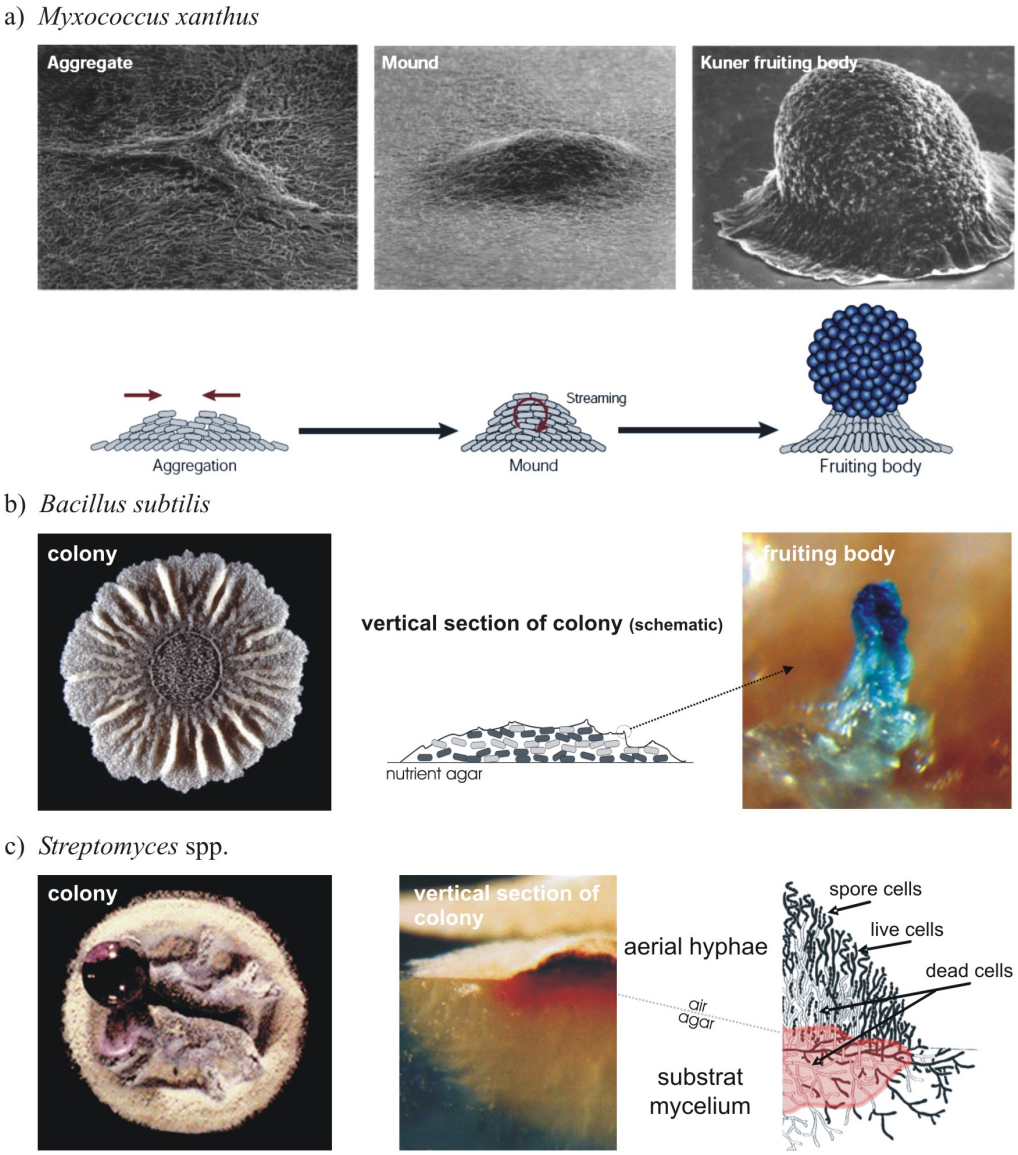

Fig. 8. Multicellular communities by *Myxococcus xanthus*, *Bacillus subtilis* and *Streptomyces* spp. *a) M. xanthus* forms multicellular communities (fruiting bodies) in which different phenotypic cell types (e.g. motile cells and non-motile cells) participate in development of the multicellular structure. Spore cells are differentiated in mature multicellular structures (see main text) (Images, modified from: Kaiser, 2003). *b)* In colony biofilms by *B. subtilis* various phenotypic cell types have been observed, e.g. motile cells, non-motile cells, matrix-producing cells, spore cells (see main text). To the left: colony formed by a wild type *B. subtilis* (Credit: Claudio Aguilar, HMS, Boston). In the middle: schematic representation of a vertical section through a colony. Some cell types can predominantly be found in distinct areas within the multicellular structure (see main text). To the right: Image from a fruiting body, which is found at the biofilm/air interface of wild type *B. subtilis* colonies. Blue dye present at the tip originates from conversion of X-Gal by β-galactosidase, expressed from the sporulation-specific promotor P*sspE* (Source: Branda *et al.*, 2001). *c)* On nutrient agar *Streptomyces* form mycelial colonies, which contain various cell types (see main text). To the left: colony formed by *Streptomyces coelicolor* A3(2) on a nutrient agar (Credit: John Innes Centre, Norwich, UK). In the middle: Vertical section through a *Streptomyces* colony (Credit: Jamie Ryding, Norwich, UK). To the right: schematic representation of the vertical section: chains of black circles represent spore cells, black hyphae represent live cells, white hyphae represent dead cells, red area represents the zone of antimicrobial compounds released into the immediate environment (Source, with modifications: Chater, 2006).

2.2 Characteristics of cell types in relation to cell populations

As described in the section above (and chapter 3), when laboratory-grown multicellular communities are looked at through a microscope, supported by advanced techniques, a remarkable spatial organization is apparent. Not all cells inhabiting the clonal multicellular community are alike. Instead, cells situated in one area of the multicellular structure differ significantly from cells that are present in another area. Their phenotype can differ with respect to metabolic/physiological activity, gene expression profiles, productivity of extracellular (macro-)molecules, responsiveness to various environmental stimuli, and sensitivity to toxic compounds (see also chapter 3). Cells, which exhibit the same phenotype are often found to be spatially co-localized resulting in the formation of distinct subpopulations. Nevertheless, it seems as that dependencies between the different cell subpopulations in a microbial community exist, presumably also at the level (or because) of metabolic interactions, which impact on the differentiation of subpopulations and organization of the entire community. Metabolic interactions and their effect on spatial organization of a microbial community have been described for laboratory-grown mixed two-species microbial communities. In these mixed communities the two species were found to organize spatially to optimize metabolic interaction. Moreover, differentiation into phenotypic subpopulations was observed for one of the two species driven by metabolic interaction with the second species (Christensen *et al.*, 2002; Nielsen *et al.*, 2000). Similar and even more complex differentiation processes and spatial organizations are likely to occur in natural multi-species microbial communities.

Differentiation into phenotypic cell types implicates that these processes are reversible, and that cells can switch between different phenotypes as a result of an adaptation response to changes occurring in their immediate surrounding environment. In some cases, however, differentiation into another cell type is manifested due to genetic variations that occurred in cells. Various factors are assumed to play role in causing and maintaining a genetic variation in a (sub-) population, but the general notion is that stress factors play a major role (Rosenberg, 2001; Tenaillon *et al.*, 2004). These stress factors might either i) be generated by the participating members of a microbial community themselves due to unbalanced metabolic interactions, so that toxic metabolic intermediates accumulate as they are not processed further, or ii) act from the environmental surrounding on the cells, such as compounds of the innate immune defence, or antimicrobial agents. Therefore, one could speculate that in microbial communities, in which the interactions between the participating cells and/or the interactions of the cells with their immediate environmental surroundings are unbalanced so that stress acts on the microbial cells, genetic variations are likely to occur and to maintain (e.g. *P. aeruginosa* cell populations during chronic lung infection in CF patients). However, in adapted microbial communities in which the interactions and dependencies between the participating microbial cells and their surroundings are well regulated and balanced, the occurrence and maintenance of genetic variations can be expected to be lower (e.g. in mutually beneficial microbial communities inhabiting the human host).

The co-existence of a variety of different phenotypic cell types and their spatial organization into confined subpopulations might be characteristic for all microbial multicellular communities. This does, however, not imply that the factors and processes that modulate cellular differentiation, assembly, and structural organization of the various multicellular communities are the same ones. For example, the development of fruiting bodies by Myxobacteria is commonly described to follow a confined genetic program under

a variety of environmental (starvation) conditions. The formation of mushroom-like structured biofilms by *P. aeruginosa*, or wrinkly colony biofilms by *B. subtilis* on the other hand, appear to be strongly dependent on environmental conditions and therefore might reflect more an optimized adaptation process to establish in a particular ecological niche. It can be assumed that also the formation and composition of various microbial communities in nature are largely driven by the prevailing environmental factors and in addition depend on the inherent metabolic capacities of the participating cells. Nevertheless, local microenvironments within multicellular structures, generated by the participating cells themselves, will impact on structural organization of all multicellular communities. Overall, it seems as that the spatial assembly and organization of microbial multicellular communities is driven by the prevailing environmental conditions in a particular niche, the inherent characteristic traits of the participating cells and local microenvironments within the multicellular structure.

As mentioned in the beginning of this chapter, global analysis on populations of biofilm cells and populations of planktonic cells revealed significant differences between these populations at the transcriptomic and proteomic levels. Can these differences be explained by supposing that results obtained from planktonic cell populations reflect one physiological status (e.g. exponential or stationary phase), whereas results obtained from biofilm cell populations represent averages of various physiological states? I assume in the main this might be the case, however, an increasing number of investigations reveal that also in planktonic cell populations not all cells are alike. For example, planktonic exponential phase cell populations of *B. subtilis* contain at least two distinct phenotypic cell types: non-motile chains of cells ('sessile cells') and motile cells (Fig. 9a). Moreover, planktonic stationary phase cell populations of *B. subtilis* can contain mixed populations either of motile cells, endospore cells, and cannibal cells, or motile cells and competent cells, dependent on the composition of the cultivation medium (Gonzalez-Pastor *et al.*, 2003; Kearns and Losick, 2005; Maamar and Dubnau, 2005; Msadek, 1999). Another example is persister cells of *E. coli* that can be formed in exponential phase cell popu-

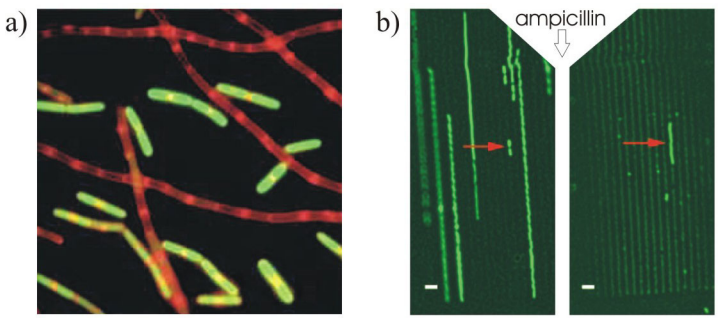

Fig. 9. Cell types in planktonic exponential phase cell populations. *a)* In exponential phase populations of *B. subtilis* laboratory strains two major cell types can be found: chains of cells (also called 'sessile cells') and motile single (doublet) cells. All cells here contain the reporter fusion P*hag-gfp*. The *hag*-gene encodes for the flagellin protein and can be regarded as marker gene for motile cells. Consistent with this only the single (doublets of) cells exhibit a green fluorescent signal. All cells were stained with the membrane stain FM4-64 (red) (Source, with modifications: Kearns and Losick, 2005). *b)* Exponential phase *E. coli* cell population examined via microfluidic technique and microscopy. Most cells of an exponential phase *E. coli* cell population are lyzed by the action of ampicillin (target: peptidoglycan synthesis) within 4 hours. However, one subpopulation of cells, so-called 'persisters', survive the ampicillin-treatment, because these cells had arrested growth prior to ampicillin-exposure. Once the antibiotic agent vanishes, the persister cells resume growth (to the right) (Source, with modifications: Balaban *et al.*, 2004).

lations (type II persisters). These cells enter a state of slow or no growth, in which they are tolerant to sudden employed antibiotic agents such as ampicillin. Once the antibiotic vanishes, the cells resume growth (Fig. 9b) (Balaban *et al.*, 2004). These are just a few of the many examples providing evidence that planktonic cell populations are by no means homogeneous cell populations as they can consist of a number of different phenotypic cell types. However, in well-shaken planktonic cell cultures all cells will experience the same environmental conditions; therefore the diversity of cell types can be expected to be lower compared to biofilm cell populations where numerous micro-environments can exist. Overall, global analysis on planktonic cell populations will represent average profiles of cells living in one niche and which under these conditions exhibit stochastic expression rates (also referred to as 'noise', e.g. Rao *et al.*, 2002), which can lead to the differentiation of distinct cell subpopulations. Global analysis on biofilm cell populations will represent average profiles of cells living in various niches and which under these conditions will exhibit different expression profiles. In addition, also possible stochastic fluctuations in gene expression will impact on the overall expression profile of biofilm cell populations.

3 *P. aeruginosa* – a biofilm model organism

This chapter aims at introducing the organism *P. aeruginosa* and to provide insights into the biofilm life style of *P. aeruginosa*. At first, the principal constituents of *P. aeruginosa* will be presented, which allow this organism to interact with its environment and form distinct microbial communities. Thereafter, the development of the mushroom-shaped biofilm by *P. aeruginosa* will be described. At last, knowledge derived from studies involving antimicrobial treatments on *P. aeruginosa* biofilms will be presented.

3.1 *P. aeruginosa* and its basic elements

P. aeruginosa has become a model organism in microbiology and in particular in biofilm biology. It belongs to the γ-Proteobacteria and is a Gram-negative polar flagellated and piliated rod-shaped bacterium with a high GC-content of about 67%. In nature, *P. aeruginosa* can be isolated from various environmental habitats, such as terrestrial and aquatic niches as well as from animal and plants (e.g. Ramos, 2004). It is easily cultivable in the laboratory because it has no special nutritional requirements and it can multiply in reasonable time at temperatures ranging from ~14-42ºC. Cultures of *P. aeruginosa* have a typical green-bluish color due to the production of phenazines. For this trait it has been named *P. aeruginosa*, for the Latin *aerugo*, which refers to the blue-green rust of copper (Price-Whelan *et al.*, 2006).

P. aeruginosa as opportunistic pathogen

P. aeruginosa has raised increasing attention as it can cause a wide range of opportunistic human infections. People afflicted with cystic fibrosis or individuals suffering from cancer, AIDS, burn wounds and patients in intensive care units frequently acquire infectious diseases caused by *P. aeruginosa*. Among those infections are pulmonary infections, medical-device-related infections, urinary tract infections, wound infections, and bacteremia (Bodey *et al.*, 1983; Ramos, 2004). In particular pulmonary infections in cystic fibrosis patients, medical-device associated infections and wound infections have been associated with the biofilm lifestyle of *P. aeruginosa*. (e.g. Høiby *et al*., 2001; Hall-Stoodley *et al*., 2004; Harrison-Balestra *et al.*, 2003). Generally it appears as the pathogenic trait of *P. aeruginosa* is not restricted to humans, since this organism can cause disease in a great variety of eukaryotic organisms, e.g. *Mus* spp., *Arabidopsis thaliana*, *Caenorhabditis elegans, Drosophila melanogaster*, *Dictyostelium discoideum*, *Canis* spp., *Lactuca* spp., and *Galleria mellonella* (D'Argenio *et al.*, 2001; Elsinghorst, 2003; Mahajan-Miklos *et al.*, 2000; Pukatzki *et al.*, 2002; Rahme *et al.*, 2000).

The genome

The genome size of *P. aeruginosa* can vary and range from 5.2 to 7 Mbp (Tümmler, 2006). It appears that the genomes of *P. aeruginosa* clones are made up of clonal typical core genome segments and accessory genome segments as well as of blocks in the core genome, which originate from unrestricted gene flow within the population (Tümmler, 2006; Shen *et*

al., 2006; Spencer *et al.*, 2003; Wiehlmann *et al.*, 2007; Wolfgang *et al.*, 2003). The accessory genome consists of a variable set of genomic islets and genomic islands (e.g. PAPI-1, PAGI-2). Moreover the genome can harbor the sequences of prophages. A finished genome sequence for one *P. aeruginosa* strain, namely PAO1 (6.3 Mbp), is available (Stover *et al.*, 2000) and deep draft genome assemblies for several other strains (e.g. PA14).

Cell surface appendages

The flagellum is a striking proteinaceous organell localized at one pole of the bacterial cell (Fig. 10). About 40 proteins are involved in flagella formation, either directly as structural components of the flagellum or as assembly components. Flagella-driven motility allows *P. aeruginosa* to actively transport itself to other locations such as in liquid environments and under laboratory conditions of semi-solid agar plates (swimming and swarming). Moreover, the flagellum has been found to be involved in adhesion and structural biofilm formation (e.g. O'Toole and Kolter, 1998b; Klausen *et al.*, 2003a; McEachran and O'Toole, 2007; see also chapter 3.2). The flagella can also be recognized by eukaryotic hosts through Toll-like receptor 5 (TLR5) and elicit an immune response (Dasgupta *et al.*, 2004). Another kind of surface appendages of *P. aeruginosa* are type IV pili (Fig. 10). PilA is the main structural component of type IV pili and about 20 further proteins are assumed to be involved in type IV pili structure and assembly (Mattick, 2002; Mattick *et al.*, 1996). Type IV pili can mediate adhesion to various kinds of surfaces and molecules (e.g. epithelial cells, plastic, glas and DNA) and translocation across surfaces via so-called twitching motility and swarming motility (Köhler *et al.*, 2000; Mattick, 2002; Mattick *et al.*, 1996; Skerker and Berg, 2001). Moreover they are involved in structural biofilm formation by *P. aeruginosa* (e.g. Klausen *et al.*, 2003a; Klausen *et al.*, 2003a; see also chapter 3.2). *P. aeruginosa* appears to produce a third kind of proteinaceous surface appendages, called cup-fimbria, which were found to be involved in biofilm formation by this organism (Vallet *et al.,* 2001).

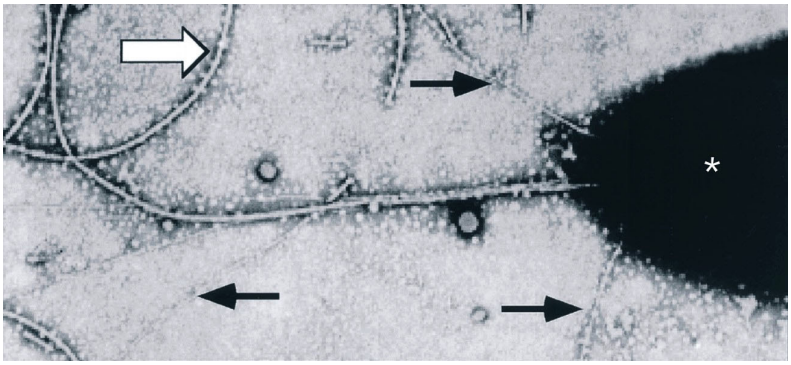

Fig. 10. Transmission electron micrograph (TEM) of a *P. aeruginosa* PAO1 cell (marked with asterisk). The white arrow points to the flagella and black arrows indicate type IV pili (Source, with modifications: O'Toole *et al.*, 2000). Type IV pili are proteinaceous filaments of about 5-6 nm in diameter, ranging from 2-5 μm in length (Folkhard *et al.*, 1981; Semmler *et al.*, 1999). Flagella can be around 4.5-9.0 μm in length and have a diameter of about 16 nm (e.g. Suzuki and Lino, 1980).

Extracellular products

P. aeruginosa can synthesize and export a variety of compounds that can be found in culture supernatants of this organism or in clinical specimens in which *P. aeruginosa* is present. Among those are small chemical molecules, as well as proteins, and polysaccharides. *P. aeruginosa* produces a mixture of molecules which exhibit surfactant-like properties. The most abundant compounds are hydroxyalkanoic acids (HAAs), and mono- and di-rhamnolipids (Fig. 11a) (Deziel *et al.*, 1999; Rendell *et al.*, 1990). Their biosynthesis is derived from central metabolic pathways, such as fatty acid biosynthesis and

dTPD-activated sugars synthesis (Fig. 11b) (Maier and Soberon-Chavez, 2005). The production of these biosurfactants was found to promote biodegradation by *P. aeruginosa* of slightly soluble organic compounds, such as aliphatic and aromatic hydrocarbons (Al-Tahhan, *et al*., 2000; Herman, *et al*., 1997; Miller, 1995; Zhang and Miller, 1994). Due to their ability in reducing surface tension the biosurfactants can also facilitate the translocation along surfaces by *P. aeruginosa* such as can be observed under laboratory conditions in semi-solid agar plates (swarming and twitching) (Caiazza *et al.*, 2005; Köhler *et al.*, 2000; Pamp and Tolker-Nielsen, 2007). In case of swarming motility the various compounds of biosurfactans can exert different effects on cellular migration and thereby modulate the structural pattern of the swarm zone (Caiazza *et al.*, 2005; Tremblay *et al.*, 2007). Furthermore, biosurfactants appear to have multiple roles in structural biofilm development by *P. aeruginosa* (see chapter 3.2). With regard to human infections the biosurfactants are found to possess effects on the respiratory tract by perturbing mucocilliary clearance and releasing mucus glycoconjugates (Read, *et al*., 1992; Somerville, *et al*., 1992). Moreover, biosurfactants seem to affect host cells, e.g. by priming oxidative burst response and phagocytic response of monocytes but also by inducing necrosis of polymorphonuclear leukocytes (PMNs) (Jensen *et al.*, 2007; Kharami *et al*., 1989; McClure and Schiller, 1992). Furthermore, the biosurfactants were shown to increase the solubility and bioactivity of another exported molecule produced by *P. aeruginosa*, namely *Pseudomonas* quinolone signal (PQS) (Calfee *et al.*, 2005). PQS together

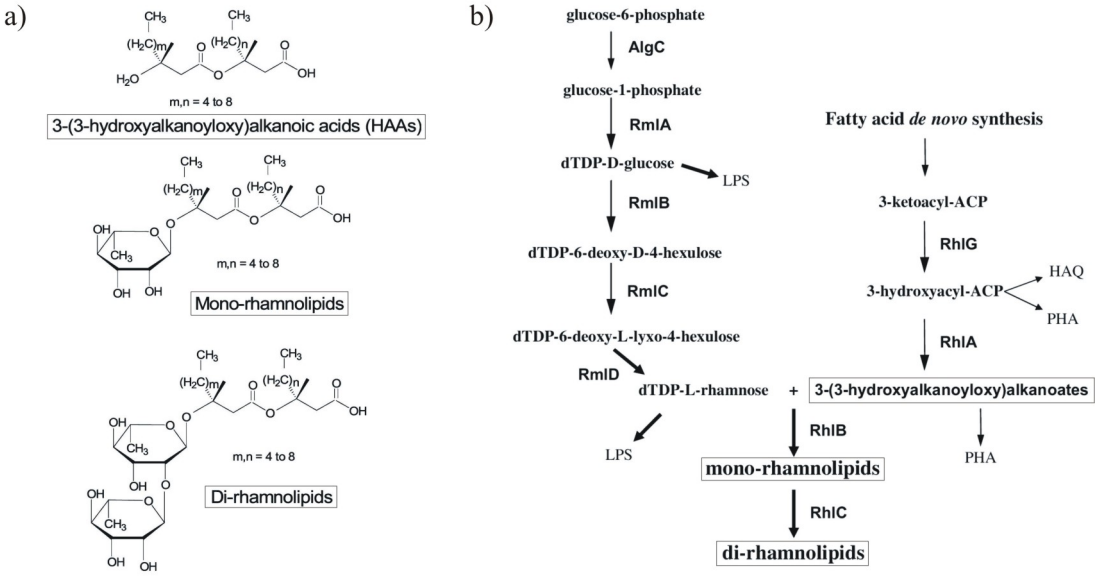

Fig. 11. Biosurfactants produced by *P. aeruginosa*. *a) P. aeruginosa* produces a mixture of biosurfactant-like molecules of three major structural groups: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), mono-rhamnolipids, and di-rhamnolipids. The alkyl-chains of these congeners can range from C_8 to C_{12}, however, the most abundant congeners under laboratory conditions have C_{10}-alkyl-chains. *b)* The biosynthesis of these molecules is derived from central metabolic pathways, such as fatty acid biosynthesis and deoxythymidine diphosphate (dTPD)-activated sugars synthesis. RhlA is the enzyme responsible for the synthesis of HAAs. The rhamnosyltransferase RhlB uses dTDP-L-rhamnose and HAAs as precursors and synthesizes mono-rhamnolipids. The mono-rhamnolipids together with HAAs are the substrates for RhlC, which synthesizes di-rhamnolipids (Source, with modifications: Maier and Soberon-Chavez, 2005).

with the N-acylhomoserine lactones 3-oxo-C12-HSL and C4-HSL mediate intercellular signalling (quorum sensing) by *P. aeruginosa*. Other small chemical molecules, which can be found in the extracellular milieu of *P. aeruginosa*, and which can be produced in

response to intercellular signalling, are hydrogen cyanide and the phenazines (e.g. pyocyanin). Moreover, quorum sensing-regulated proteins such as LasA protease, alkaline protease, exotoxin A, LasB elastase, together with the lectins LecA and LecB can be released by *P. aeruginosa* into the extracellular environment (e.g. Diggle *et al.*, 2006; Price-Whelan *et al.*, 2006; Schuster and Greenberg, 2006). *P. aeruginosa* has been found to produce at least three different polysaccharides: PEL, PSL and alginate. Whereas PEL and PSL have been found to be involved in *in vitro* biofilm formation by *P. aeruginosa*, alginate predominantly is involved in *in vivo* biofilm formation by *P. aeruginosa* during chronic colonization of lungs of cystic fibrosis patients (e.g. Branda *et al.*, 2005; Ma *et al.*, 2006; Pamp *et al.*, 2007; Ramsey and Wozniak, 2005).

The cell envelope

The cell envelope, comprising inner and outer membrane and the peptidoglycan layer, separates the inside from the outside of the cell and functions as a permeability barrier. Lipopolysaccharide (LPS) is the major component of the outer membrane and can be recognized through Toll-like receptor 4 (TLR4)-MD2-CD14 complex in humans resulting in elicitation of an immune response. Dependent on environmental conditions the structural composition of LPS can be modulated. It has been observed that the lipid A part of LPS molecules of clinical cystic fibrosis (CF) isolates of *P. aeruginosa* predominantly is hexa- or hepta-acetylated, compared to lipid A of environmental *P. aeruginosa* isolates, which predominantly is penta-acetylated (Miller *et al.*, 2005). Under conditions of low concentrations of divalent cations (e.g. Mg^{2+}, Ca^{2+}), or in the presence of antimicrobial peptides (e.g. polymyxins) or polyamines (e.g. spermidine), LPS can be modified by addition of aminoarabinose to the phosphate moietys of lipid A (Fig. 12a). This modification decreases the overall negative net charge of the bacterial outer surface, and e.g. hence the interaction with antimicrobial peptides (Ernst *et al*., 1999; Kwon and Lu, 2006; McPhee *et al*., 2003). The cell envelope contains various proteins of which many are involved in the transport of molecules in or out of the cell. Porins, located in the outer membrane, mediate the uptake of a number of compounds ranging from small nutrient molecules to larger iron-siderophore complexes. Among these are general porins such as OprF and OprG as well as specific porins, such as OprB (transport of glucose and other monosaccharides), OprP (transport of phosphate), and OprD (transport of arginine and lysine) or gated porins, such as FpvA (a pyoverdine receptor) (Tamber and Hancock, 2003; Tamber and Hancock, 2004). Many antibiotics enter the cell through porins as well. A number of efflux and secretion systems are responsible for exporting a variety of small molecules from the cell, which might have toxic effects. Multidrug efflux systems of all known families have either been described or homologues identified in *P. aeruginosa*, though examples of the resistance-nodulation-division (RND)-efflux pumps (e.g. MexAB-OprM, MexCD-OprJ, MexGHI-OpmD) are the best described ones (e.g. Fig 12b). Together these efflux-pumps are able to export a great variety of substrates such as β-lactams, fluoroquinolones, triclosan, ethidium bromide, macrolides, chloramphenicol, tetracycline, or N-acylhomoserine lactones (Evans *et al.*, 1998; Poole, 2005; Schweizer, 2003). A recent study indicates that also tolerance to an antimicrobial peptide (polymyxin E) can be mediated by MexAB-OprM under conditions when *P. aeruginosa* is living as biofilm (Pamp, *et al.*, submitted). As is basic to all bacteria proteins are present in the inner membrane, which participate in generating the proton motive force (PMF). The inner membrane is also the location where external signals can be received and transferred to signal transduction systems (see next section) inside the cell. *P. aeruginosa* is able to degrade a wide range of organic compounds and consistent with this observation it has been found that *P. aeruginosa* is attracted to a great variety of different compounds, such as amino acids, sugars, phospholipids, organic acids, aromatic acids and oligopeptides (e.g.

Parales *et al.*, 2004). Signal-reception of small molecules occurs for example through methyl accepting proteins (MCPs) involved in chemotaxis. Environmental signals can also be received through histidine-sensor kinases (HisKin), which are members of phosphor-transfer signal transduction systems. The genome of *P. aeruginosa* PAO1 encodes for 26 MCPs (e.g. McpA, McpB, PilJ) and 63 HisKin (e.g. PmrB, PhoQ) (Galperin, 2005).

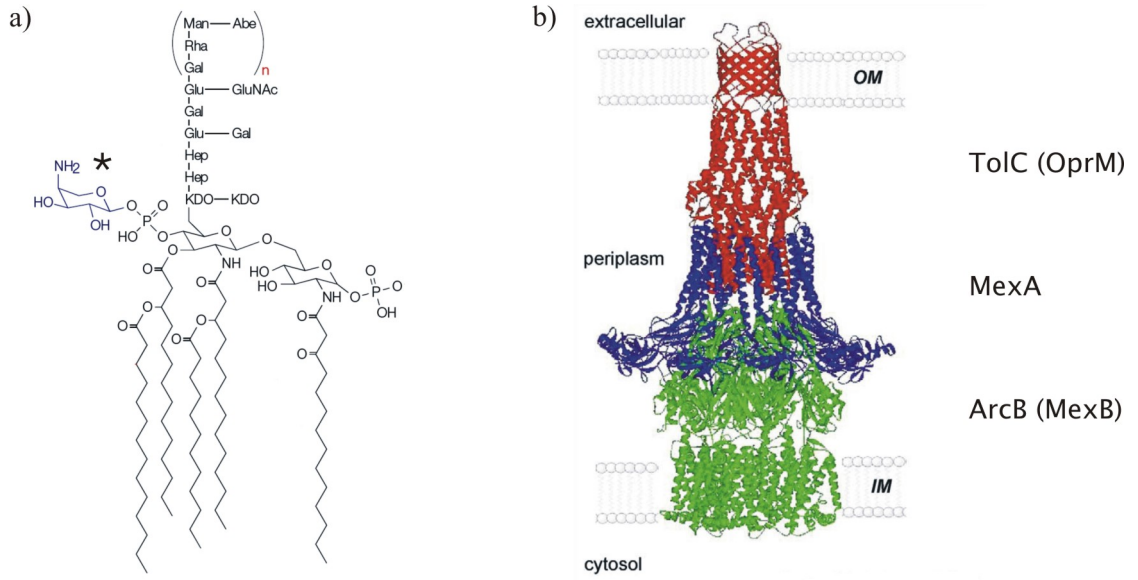

Fig. 12. Components of the cell envelope, which can confer resistance or tolerance to antimicrobial compounds. *a)* Chemical structure of lipopolysaccharide (LPS) with 4-amino-4-deoxy-L-arabinose (marked with asterisk) bound to one phospate residue of lipid A (hier: LPS from *Salmonella enterica* serovar Thyphimurium as example) (Source, with modifications: Miller *et al.*, 2005). *b)* Model of an assembled efflux pump of the resistance-nodulation-division (RND) family. ArcB (homologous to MexB from *P. aeruginosa*) (green) constitutes the energy-providing, substrate-binding component in the inner membrane. MexA (blue) is a periplasmatic 'adaptor', linking the inner and outer membrane components. TolC (homologous to OprM from *P. aeruginosa*) (red) constitutes an exit duct for the substrates (Source, with modifications: Eswaran *et al.*, 2004).

Intracellular components

Remarkable complex regulatory networks control basic cellular processes such as replication and metabolism as well as processes, which govern flagella- and type IV pili-driven motility, the synthesis and export of small chemical compounds and proteins, the production of polysaccharides, and modifications of cell-surface compounds. All these processes occur as a result of an adaptation in response to local environmental stimuli such as nutrients, temperature, O_2, oxidative stress, secondary metabolites and other chemical molecules. The genome of *P. aeruginosa* PAO1 contains a high proportion of genes, which can be assigned regulatory functions. Around 517 (~9%) of all genes encode for proteins with motifs of transcriptional regulators or members of two-component systems coordinating phosphotransfer signal transduction (Kulasekara and Lory, 2004; Stover *et al.*, 2000). Genome sequence analysis also revealed the presence of 33 GGDEF-motifs and 21 EAL-motifs in proteins of *P. aeruginosa*, which are thought to be involved in regulating intracellular levels of the signal-messenger molecule c-di-GMP. In addition 24 genes have been identified which encode for putative sigma (σ) factors (Kulasekara and Lory, 2004; Stover *et al.*, 2000). Five homologues of chemotaxis gene clusters (cluster I-V) have been identified in the genome sequence of *P. aeruginosa*. Chemotaxis gene clusters encode for proteins, which mediate signal transduction such as CheA (sensor kinase), CheY (response

regulator), CheW (coupling protein), CheB (methylesterase), CheR (methyltransferase), CheZ (phosphatase) and some MCPs. Cluster I and cluster V appear to be involved in regulating flagella-driven motility. Cluster IV has been found to regulate type IV pili-driven motility, however, a recent study provides data, which indicate that this cluster might also modulate flagella-driven motility. Cluster III has been found to regulate the production of the polysaccharides PEL and PSL via c-di-GMP-signaling. The role of the chemotaxis cluster II is not yet clear, however, it seems as it might be involved in chemotactic processes of stationary phase growth by *P. aeruginosa*. With the exception of cluster II, all other four clusters have been found to be involved in biofilm formation by *P. aeruginosa* using different experimental setups (Caiazza *et al.*, 2007; Ferrandez *et al.*, 2002; Hickman *et al.*, 2005; Parales *et al.*, 2004; Whitchurch *et al.*, 2004; Barken *et al.*, in prep.). Quorum sensing is well known to orchestrate the production of extracellular chemical molecules and proteins (e.g. biosurfactants, cyanide, LasA protease, alkaline protease, exotoxin A, LasB elastase). The direct impact of quorum sensing on cellular motility and biofilm formation appears to be controversial and might be dependent on environmental conditions (e.g. Shrout *et al.*, 2006).

3.2 The development of a biofilm

As described in chapter 1.3.1, flow-chamber-grown biofilms are most suitable for detailed studies concerning biofilm development, anatomy and physiology. In this section an overview of the developmental processes and biology of *P. aeruginosa* flow-chamber-grown biofilms will be presented. It will be focused on the mushroom-like shaped biofilm, which is preferably formed under low shear force conditions when glucose is provided as carbon source. Where indicated, knowledge derived from studies on flat-structured flow-chamber-grown biofilms and colony-grown biofilms by *P. aeruginosa* will be presented. The observed phenotype of the mature *P. aeruginosa* biofilm, at a glance, is depicted as color-coded graphical fingerprint at the end of this section (Fig. 14).

Contacting the substratum

In liquid environments *P. aeruginosa* is able to live planktonically and move around by flagella-driven motility. Contact to the substratum by a fraction of planktonic cells can be initiated either passively, due to gravity or Brownian motion, or actively, by flagella-driven motility (McEachran and O'Toole, 2007). Within the first ~4 hours, at least two major phenotypic cell types at the air/liquid interface can be observed: cells that stay attached and cells that had attached but detach again. Little is known about the detached planktonic cells, but it seems as that many of those cells will be transported away by the liquid flow. Some of the attached cells can be observed bound to the surface via the cell-pole and rotating around their polar axis (e.g. Caiazza and O'Toole, 2004; Sauer *et al.*, 2002). Microscopic observations, comparing wild type strain and mutant strains, indicate that flagella and SadB might be involved in this phenotypic behavior (Caiazza and O'Toole, 2004; Sauer *et al.*, 2002). However, after ~5 hours most cells are attached to the substratum via the longitudinal side (Sauer *et al.*, 2002; Singh *et al.*, 2002; Pamp, unpublished observation). Recent research indicates that the polysaccharide PSL, which was found to surround single cells, can facilitate firm attachment of cells to the substratum (Ma *et al.*, 2006; Ma *et al.*, 2007a; Matsukawa and Greenberg, 2004).
Observations involving time-lapse CLSM indicate that the following initial developmental step at the substratum involves two classes of phenotypic cell types: non-motile cells and motile cells. Moreover, cell division is initiated. Non-motile cells can divide and the progeny cells can either be non-motile as well or induce surface-associated migration.

Motile cells can slow-down surface-associated migration and induce cell division. The progeny cells can then move along the surface or stay at the location of their origin (Fig. 13a) (Klausen *et al.*, 2003b; Shrout *et al.* 2006; Singh *et al.*, 2002; Pamp, unpublished observation). It appears that the relative abundance of these cell types and processes of cell division are delicately balanced and that a shift in favor of a certain cell type can have profound impact on the following developmental steps and the final three-dimensional structure of the biofilm. However, little is known about the cellular differentiation processes that lead to these different phenotypic cell types and their exact relative abundance. Detailed research, resolved at the single-cell level, is required to understand the intrinsic and extrinsic factors that govern these cellular differentiation processes.

Initiation of structural biofilm formation

High local abundance of non-motile and dividing cells can give rise to clonal microcolony formation (Fig. 13b), as can be observed by studying the behavior of cells (e.g. Gfp-tagged cells or mixtures or Yfp- and Cfp-tagged cells) by time-lapse CLSM (Fig. 13c) (Klausen *et al.*, 2003b; Singh *et al.*, 2002). Factors that facilitate firm attachment between the cells within these microcolonies appear to be extracellular DNA, biosurfactants, and the polysaccharide PSL. Treatment of initial microcolonies with DNase I was found to dissolve the microcolonies and it was suggested that the extracellular DNA might function as cell-to-cell interconnecting component (Whitchurch *et al.*, 2002). Cells, which are unable to produce biosurfactants (e.g. *rhlA*-mutant cells), are unable to establish firm cell-to-cell association upon local cell proliferation (Pamp and Tolker-Nielsen, 2007). The supporting effect of biosurfactants on microcolony formation might be explained by changes in cell-surface hydrophobicity, which can evidently be caused by the surface-active agents produced by *P. aeruginosa* and which might increase the adhesiveness between cells (Al-Tahhan *et al.*, 2000; Herman *et al.*, 1997; Zhang *et al.*, 1994). In support of the involvement of biosurfactants in microcolony formation it has been observed that expression of *rhlA* is induced in microcolonies of wild type *P. aeruginosa* (Lequette and Greenberg, 2005). In addition, the polysaccharide PSL appears to be involved as well as a mutant defective in PSL-production was affected in microcolony formation (Jackson *et al.*, 2004; Matsukawa and Greenberg, 2004). Using a fluorescently labeled lectin and CLSM, the polysaccharide PSL was found to be located in the upper layers of microcolonies formed by the wild type and it is thought that PSL functions as a scaffold, holding the biofilm cells together in the microcolony (Ma *et al.*, 2007b). The production of PSL in biofilms seems to be regulated via the intracellular messenger c-di-GMP, which relative amounts appear to be modulated by gene products of the chemosensory cluster III (Wsp-cluster) (Hickman *et al.*, 2005).

Within the same time frame of initial microcolony formation, an increasing number of motile cells can be observed migrating along the surface of the substratum (Fig. 13b). This process appears to be facilitated by type IV pili, as mutant cells defective in type IV pili formation or function (e.g. *pilA*-, *chpA*-, *pilJ*-, *pilK*-mutants) exhibit no or decreased surface-associated motility, as can be observed by time-lapse CLSM (Klausen *et al.*, 2003b; Pamp, unpublished observation). In contrast, these strains exhibit somewhat prematurely microcolony formation relative to the wild type, presumably due to the absence or low number of motile cells (Pamp, unpublished observation). The opposite is true in cases where surface-associated motility is stimulated, such as under conditions of low concentrations of iron or the presence of citrate as carbon source instead of glucose. Singh *et al.* (2002) showed that in the presence of iron-chelating lactoferrin, surface associated type IV pili-driven motility is increased and microcolony formation prevented. Klausen *et al.* (2003a) observed that when citrate is present, cells spread out from initial microcolonies resulting in a flat-structured biofilm. Comparative analysis involving the wild type strain,

pilA- and *fliM*-mutants suggested that cells spread largely driven by type IV pili and to a minor degree by flagella-driven motility (Klausen *et al.*, 2003a).

Structural biofilm maturation

Both subpopulations of cells, the microcolonies formed by non-motile cells, and the cells exhibiting surface-associated motility, are originators for further steps in development of the mushroom-like structured biofilm. Biofilm analysis involving single strains (e.g. Gfp-tagged) or mixed strains (e.g. Cfp-tagged + Yfp-tagged strains), in combination with CLSM, time-lapse CLSM and/or FRAP (fluorescence recovery after photobleaching)-CLSM revealed that formation of the mushroom-shaped biofilm occurs by climbing of motile cells and proliferation of these cells on top of microcolonies formed by non-motile cells (Fig. 13c). The microcolony-forming subpopulation is thereafter also referred to as 'stalk' and the subpopulation on top has been referred to as 'cap'.

Time-lapse FRAP-CLSM analysis on a 2-day-grown biofilm by the wild type strain revealed the presence of motile cells surrounding the microcolony formed by non-motile cells (Haagensen *et al.*, 2007). Mixed color-coded time-lapse CLSM involving non-motile cells (e.g. Cfp-tagged *pilA*-mutant) and motile cells (e.g. Yfp-tagged wild type) revealed, that microcolonies are largely formed by the *pilA*-mutant whereas wild type cells are migrating along the substratum and colonize the top of the microcolonies to form the cap-forming subpopulation (Klausen *et al.*, 2003b). Generally, three factors have been found to be involved in surface associated motility by *P. aeruginosa*: type IV pili, biosurfactants and flagella. Recent studies indicate that all three factors contribute to different degrees in facilitating migration of cells up on the microcolonies. As *pilA*-mutant cells are not able to climb up on a microcolony it has been thought that type IV pili-driven cellular migration is the main factor involved in establishment of a cap-forming subpopulation (Klausen *et al.*, 2003b). In support of this, *pilA*-gene expression was found induced in the fraction of cells that colonize the top of microcolonies (Barken *et al.*, unpublished observation). However, studies involving mutants, which express to some degree type IV pili, but are affected in function of type IV pili, such as chemotaxis cluster IV-mutants (e.g. Δ*chpA*, Δ*pilJ*, Δ*pilK*) or a *fimL*-mutant were found to be able to climb on microcolonies formed by a *pilA*-mutant (Whitchurch *et al.,*2005; Barken *et al*., in prep.; Pamp, unpublished observation). This suggests a minor role for type IV pili-driven motility in cap-formation. But as the caps formed by these mutant strains were irregular-shaped compared to the smooth cap formed by wild type cells, it seems that type IV pili-driven motility and/or chemotaxis might be involved in modulating the three-dimensional structure of the cap-forming subpopulation (Whitchurch *et al.,*2005; Barken *et al*., in prep.; Pamp, unpublished observation). Since type IV pili are essential for formation of a cap-forming subpopulation (Klausen *et al.*, 2003b), it is hypothesized that the type IV pili are rather required for attachment and binding of motile cells to the microcolony, than required for facilitating cellular migration. This idea is originated from the observation that type IV pili can bind DNA, and extracellular DNA has been found to cover microcolonies of the wild type strain (Allesen-Holm *et al.*, 2006; Van Schaik *et al.*, 2005).

To examine the impact of biosurfactants produced by *P. aeruginosa* on facilitating migration of the motile cells to the top of the microcolonies, investigations have been performed on biofilms initiated with Cfp-tagged *pilA*-mutant cells and Yfp-tagged *rhlA*-mutant cells. In these experiments some *rhlA*-mutant cells were able to climb onto the microcolonies, but the final cap-forming subpopulation was significantly reduced in size compared to the caps formed by the wild type cells (Pamp and Tolker-Nielsen, 2007). This suggests, that biosurfactants can facilitate migration of motile cells on the microcolonies,

presumably by mechanisms that reduce surface tension. An additional role is suggested for biosurfactant production during later stages of structural biofilm maturation. In this case biosurfactants seem to be responsible for keeping the water channels in between the mushroom-like structures open (Davey *et al.*, 2003).

Recent investigations aimed at determining the role of flagella in facilitating cellular migration and colonization of microcolonies indicate that these surface appendages play a major role in cap-formation. Experiments carried out on mixed-strain biofilms involving Cfp-tagged *pilA*-mutant cells and Yfp-tagged *fliM*-mutant cells revealed that the *fliM*-mutant is affected in development of a cap-forming subpopulation. Only a few cells were able to colonize on top of the microcolonies (Barken *et al.*, in prep.). Together this suggests that in initial stages of the maturation phase motile cells might attach to the microcolonies via type IV pili and that the colonization of cells on top of the microcolonies is facilitated by flagella-driven motility supported by the secretion of surface-active agents. It is not clear whether in later stages of the maturation phase the cells forming the cap are still actively migrating, as this is difficult to examine due to technical limitations of CLSM. Initial attempts to address this issue, however, suggest that most cells of the cap-forming subpopulation are not moving during late stages of biofilm maturation (Pamp, unpublished observation).

During all stages of biofilm maturation the cells in the upper layer of the microcolony/mushroom-like structure exhibit a higher metabolic activity compared to the cells inside these structures (Pamp *et al.*, submitted). This can be explained by the fact that concentrations of dissolved O_2 (and nutrients) are high in the bulk-liquid, and therefore easily accessible to the cells situated in the upper layer of the biofilm structure. High concentrations of O_2 (and nutrients) in the bulk liquid are likely to be the driving-force for cells to colonize the top of initial microcolonies, and the ability of motile cells, in contrast to non-motile cells, to transport themselves to new areas seems to be advantageous in conquering the top of microcolonies. The idea seems to be supported by a result obtained from a mixed three-color-coded experiment involving wild type cells, *rhlA*-mutant cells, and *pilA*-mutant cells: Only the wild type cells, which have the full capacity to migrate, were able to establish in the most upper layer of the multicellular structure, in contrast to the mutant cells which were affected in cellular migration, and established in the lower areas (Pamp and Tolker-Nielsen, 2007). Limited amounts of O_2 within the deeper areas of flow-chamber-grown biofilms can be expected as has been shown by microelectrode oxygen measurements on colony biofilms by *P. aeruginosa* (Werner *et al.*, 2004; Xu *et al.*, 1998). That the cells in the deeper layers of flow-chamber-grown biofilms have access to only very low amounts or no O_2 can be concluded from the observation that *nirS* (nitrite reductase), required for anaerobic respiration, is induced in cells situated in the deeper layers of the multicellular structures. In addition, reactive nitrogen intermediates (RNI) such as nitric oxide (NO), resulting from denitrification, were found to be present in the deeper areas of the biofilm (Barraud *et al.*, 2006). The cells in the deeper layers of mushroom-shaped biofilms appear also to experience iron-limiting conditions, as *pvdA*-expression is induced in these cells (Kaneko *et al.*, 2007).

Dissemination from the biofilm

In aging biofilms some cells can be observed to leave the multicellular structures and become planktonic (Fig. 13d). This process is commonly termed dispersion and in *P. aeruginosa* it can follow the so-called hollowing pattern. The term ‘hollowing’ results from observations in which void spaces (hollows) in the center of multicellular structures appeared. The hollows are usually filled with a great number of rapidly moving cells, which

eventually can find a way out of the hollows and become planktonic. In addition, dead cells have also been observed inside the void spaces (Barraud *et al.*, 2006; Boles *et al.*, 2005; Hunt *et al.*, 2004; Purevdorj-Gage *et al.*, 2005; Sauer *et al.*, 2002; Webb *et al.*, 2002; Webb *et al.*, 2004).

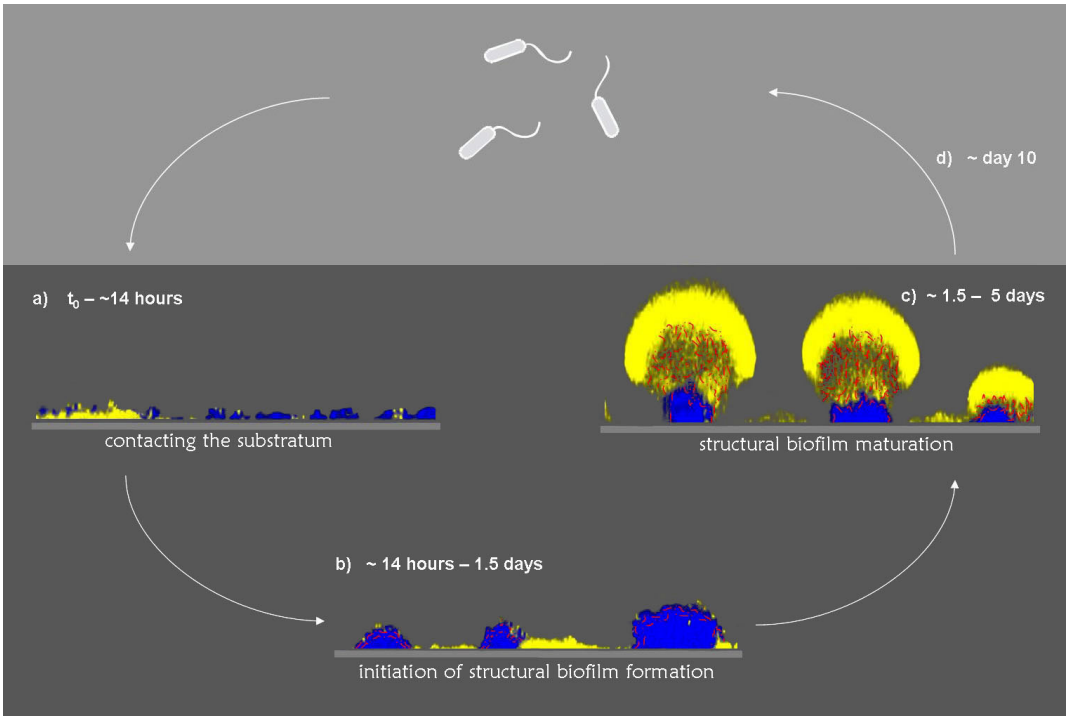

Fig. 13. Schematic representation of the structural biofilm development of the mushroom-like structured biofilm by *P. aeruginosa*. *a)* Cells get in contact with the substratum and establish firm attachment. Differentiation of the cell population into two phenotypic subpopulations: motile cells (yellow) and non-motile cells (blue). Motility is facilitated by type IV-pili-driven motility. Moreover, cell proliferation takes place. *b)* High local abundance of non-motile and dividing cells gives rise to microcolony-formation (blue). Appearance of extracellular DNA in the microcolonies (red). In addition, a second cell population migrates along the substratum (yellow). *c)* A motile subpopulation of cells (yellow) climbs up on the microcolonies (blue). Motility requires flagella, biosurfactants, type IV pili and extracellular DNA. The mature mushroom-like structured multicellular communities are composed of two major spatially distinct subpopulations: the 'cap' (yellow) and the 'stalk' (blue). *d)* In aging biofilms some cells can detach from the multicellular structures to enter the planktonic mode of life.

Different factors are reported to trigger hollowing, however, generally it appears that a certain threshold diameter of the mushroom-like structures is required (e.g. Purevdorj-Gage *et al.*, 2005). This could indicate that low local concentrations of O_2 or nutrients in the deeper areas of the multicellular structures might impact on induction of hollowing. A recent study showed that under glucose-starved continuous flow conditions hollowing and dispersion is induced (Hunt *et al.*, 2004). Sauer and colleagues describe that an increase in carbon source or shift to another carbon source could induce dispersion (Sauer *et al.*, 2002).

This suggests that generally changes in environmental conditions might be sufficient to induce dispersion. In favor of the hypothesis that low anoxic local environments could trigger hollowing, Barraud and colleagues recently described that reactive nitrogen intermediates (RNI), arising from anaerobic respiration, can induce cell-death and dispersion (Barraud *et al.*, 2006). These processes also occurred concurrently with an increased number of phages in the effluent, which might indicate that hollowing formation could be a result of phage-induced cell lysis. Investigations carried out using motility-plate

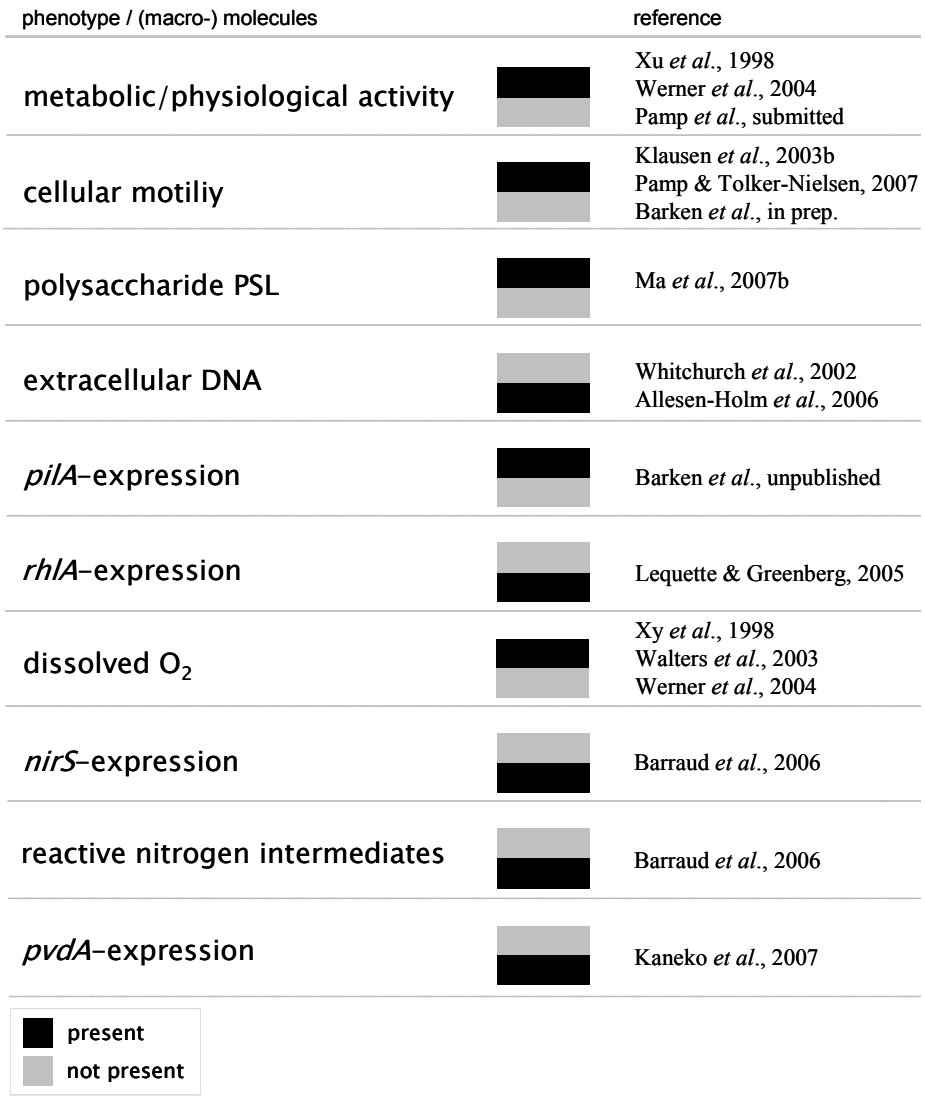

Fig. 14. Phenotype of mature *P. aeruginosa* biofilms presented schematically as color-coded graphical fingerprint. It is striking that the various described phenotypic characteristics of *P. aeruginosa* biofilms (flow-chamber-grown and colony) exhibit a distinct binary structural distribution: a phenotype is expressed in cells situated closer to the substratum but not in the cells situated in the upper area of the multicellular structure, or vice versa (see main text of this chapter). Also, macromolecules as well as small molecules appear to be present in high amounts in one area and present in low amounts in the other area. These observations provide the basis for this schematic representation. The square represents a vertical section through a biofilm, divided into two parts representing the upper and lower area of a biofilm, respectively (not to scale). *Black*: the phenotype is expressed preferably in this area / the amount of a molecule is highest in this area. *Grey*: the phenotype is not well expressed in this area / the amount of a molecule is low in this area. The representation is a simplification of the described observations from the various studies and should be regarded as such.

assays indicate, that low concentrations of NO can also stimulate swarming motility and it is speculated that NO therefore could induce motility in the surviving cells in the deeper layers of biofilms resulting in dispersion (Barraud *et al.*, 2006). In support of these observation, Sauer and colleagues observed by global transcriptomic analysis on separated dispersed and biofilm cells, that flagella and phage genes were induced in dispersed cells, whereas genes involved in denitrification were induced in biofilm cells (Sauer *et al.*, 2002). A mechanism of dispersion independent of flagella-driven motility was described recently. In this study an MCP (BdlA) and changes in intracellular levels of c-di-GMP were reported

to be required for dispersion (Morgan *et al.*, 2006). Boles *et al.* (2005) observed, that induction of *rhlAB*-expression and resulting biosurfactant production in biofilms leading to induction of hollowing and dispersion of cells from the biofilms. Moreover they showed that also biosurfactants provided from the outside had a similar effect on biofilms, indicating that cells in the interior part of the biofilm exhibited sensitivity to the surface-active agent, in contrast to the surrounding cells. Purevdorj-Gage *et al.* (2005) reported, that biofilms formed by a *rhlA*-mutant also induced hollowing and dispersion similar to the wild type strain, indicating that biosurfactants can trigger hollowing and dispersion but are not essentially required.

3.3 Antimicrobial action in biofilms

Biofilms living in the environment as well as those associated with animals and plants or present in man-made environments are frequently exposed to antimicrobial compounds, both of natural and synthetic origin. Flow-chamber-grown biofilms appear to be a useful model system to study antimicrobial action in biofilms, e.g. as the spatial appearance and distribution of dead and surviving cells in a biofilm upon antimicrobial attack can be followed in real-time. This section will give an overview of the spatial antimicrobial susceptibility and tolerance phenotypes of *P. aeruginosa* biofilms living in flow-chambers. Moreover, extrinsic and intrinsic determinants that can lead to these phenotypes will be discussed. As summary, the observed phenotypes are depicted at a glance as color-coded graphical fingerprint at the end of this section (Fig. 16).

As described in the previous section, *P. aeruginosa* biofilms are composed of two major subpopulations, a subpopulation situated close to the substratum and a subpopulation on top. Interestingly, all antimicrobial compounds tested by now, seem to exert their antimicrobial effects on either of the two subpopulations, the subpopulation close to the substratum or the subpopulation on top, whereas the other subpopulation survives the treatment. In most cases the surviving subpopulation of cells exhibits phenotypic tolerance and not resistance.

Exposure of biofilms to colistin, EDTA, SDS, novispirin G10, chlorhexidine gluconate or gallium

If *P. aeruginosa* biofilms were exposed either to colistin, EDTA, SDS, novispirin G10, chlorhexidine gluconate or gallium, the cell subpopulation situated in the interior area close to the substratum was killed, whereas the cells situated in the upper area of the multicellular structure survived the treatment.

Colistin is an antimicrobial peptide, which is administered as treatment against infections caused by Gram-negative bacteria, e.g. pulmonary infections caused by *P. aeruginosa* in CF patients. This compound is thought to exert its primary antibacterial effect through interactions with the membrane, resulting in leakage and eventually death of the bacterial cell (Hancock and Chapple, 1999; Storm *et al.*, 1977). The spatial distribution of live and dead cells appears to be independent of the actual three-dimensional structure of the biofilm and the carbon source used for biofilm-growth, e.g. as mushroom-shaped as well as irregular-shaped glucose-grown biofilms, and flat-structured wild type biofilms grown on citrate exhibited a subpopulation of dead cells close to the substratum and a subpopulation of live cells on top (Haagensen *et al.*, 2007; Pamp *et al.*, submitted; Haagensen, unpublished observation). In premature glucose-grown wild type biofilms, development of colistin tolerance in cells situated in the upper area appeared to be dependent on type IV pili-mediated cellular migration (Haagensen *et al.*, 2007). The mechanism by which type IV

pili-dependent cellular migration mediates the development of colistin tolerance in premature biofilms is unknown at present. In mature glucose-grown wild type biofilms, development of colistin tolerance in cells situated in the upper area was linked to high metabolic activity, and found to be independent of cellular migration (Pamp *et al.*, submitted). Experiments involving fluorescent *in situ* gene expression under control of a growth-rate-dependent promotor (P1*rrnB*) revealed, that colistin targets preferably biofilm cells exhibiting low metabolic activity, whereas biofilm cells exhibiting high metabolic activity survive the treatment (Pamp *et al.*, submitted). Further experiments involving fluorescent *in situ* gene expression, under control of P*pmrH* or P*mexA*, and the comparison of wild type and mutant strains, e.g. Δ*pmrF*, Δ*pmrB* , Δ*mexAB-oprM* suggested, that tolerance development to colistin in these biofilm cells is dependent on LPS-modification mediated by the *pmr*-operon, and efflux of colistin mediated by *mexAB-oprM* (Haagensen *et al.*, 2007; Pamp *et al.*, submitted).

EDTA has the ability to form complexes with metal ions, such as Mg^{2+}, Ca^{2+} and Fe^{3+}. Exposure of proteobacterial cells with EDTA has been reported to result in removal of divalent cations (Mg^{2+}, Ca^{2+}) from LPS of the outer membrane and consequently in disruption of the outer membrane (Hancock, 1984; Tamber and Hancock, 2004). Exposure of mature wild type biofilms does not only induce cell death in the subpopulation of cells close to the substratum, it also results in dispersal of a fraction of viable cells from the multicellular structures (Banin *et al.*, 2006; Pamp, unpublished observation). Induction of cell death and dispersal by EDTA could be inhibited by the addition of Mg^{2+}, Ca^{2+} or Fe^{3+} ions, supporting the notion, that EDTA exerts its effects on biofilm cells by complex-formation with metal ions, which are present in the outer membrane of the cells and possibly also part of the stabilizing extracellular matrix (Banin *et al.*, 2006). The reason for why the upper subpopulation of cells survived the EDTA treatment is unclear at present. The fact that expression of the *pmr*-operon is induced in the cell subpopulation in the upper layer suggested that tolerance to EDTA was conferred by *pmr*-mediated LPS-modification. However, the observation that *pmr*-mutants (e.g. Δ*pmrF*, Δ*pmrB*) exhibit wild type phenotype upon EDTA exposure indicate, that the tolerance mechanisms is independent of *pmr*-mediated LPS-modification (Pamp *et al.*, in prep.).

SDS is a synthetic anionic surfactant, and this compound and its derivatives can be found in many household products (e.g. soaps). Due to its amphiphilic characteristics SDS interferes with biological membranes and is also known for its ability to denature proteins. High concentrations of SDS (0.01%) induce cell death in the cell subpopulation close to the substratum in premature as well as mature wild type biofilms within 30-60 minutes (Haagensen *et al.*, 2007; Pamp, unpublished observation). Lower concentrations of SDS (0.003%) can induce cell death in the cell subpopulation close to the substratum in mature wild type biofilms within 13 hours (Pamp *et al.*, in prep.). Another study reported disruption of *P. aeruginosa* biofilm via hollowing by exposure with 0.2% SDS for 1.5 hours (Boles *et al.*, 2005). However, as the reported experiment was performed in the absence of a fluorescent indicator for dead cells (e.g. propidium iodide) it is unknown whether cells from the interior area of the biofilm detached or were killed. The genetic determinants and mechanisms, which facilitate tolerance development of the surviving fraction of cells is unknown at present. Tolerance development to SDS in the cells situated in the upper area appears to be independent of *pmr*-mediated LPS-modification, as *pmr*-mutants exhibit wild type phenotype upon exposure and the *pmr*-operon is not induced in the surviving fraction of cells (Pamp *et al.*, in prep.).

The antimicrobial peptide novispirin G10 is a synthetic alpha-helical octadecapeptide with structural similarities to sheep myeloid antimicrobial peptide 29, and its target appears to be the bacterial membrane. This peptide and its derivatives are considered as a novel potential therapeutic against infections, e.g. caused by *P. aeruginosa* (Eckert *et al.*, 2006; Jacobsen *et al.*, 2007; Sawai *et al.*, 2000; Song *et al.*, 2005; Steinstraesser *et al.*, 2002). If mature *P. aeruginosa* wild type biofilms were exposed to novispirin G10, the cells situated in the interior area were killed, whereas the cells situated in the upper area of the multicellular structure survived the treatment (Pamp *et al.*, in prep.). Preliminary observations suggest, that *pmr*-mediated LPS-modification is involved in tolerance development to novispirin G10, as expression of the *pmr*-operon is induced in the cells in the upper area of the biofilm upon exposure, and biofilms formed by *pmr*-mutants exhibit increased sensitivity to novispirin G10 (Pamp, unpublished observation).

Chlorhexidine gluconate is a synthetic antiseptic predominantly used as treatment against plaque and oral infections. It can interfere with microbial membranes, which can lead to perturbations and eventually death of the microbial cell (e.g. Vitkov *et al.*, 2005). If mature *P. aeruginosa* wild type biofilms were exposed to chlorhexidine gluconate, the cells situated in the interior area were killed, whereas the cells situated in the upper area of the multicellular structure survived the treatment (Pamp *et al.*, in prep.). The genetic determinants and molecular mechanisms, which are required for tolerance development to chlorhexidine gluconate in this particular subpopulation of cells is unknown at present.

Gallium nitrate ($Ga(NO_3)_3$) is used as treatment in cancer therapy. It can inhibit growth of various lymphoma cell lines by interfering with cellular iron metabolism. The Ga^{3+}-ion strongly resembles the Fe^{3+}-ion and many biological systems are unable to distinguish Ga^{3+} from Fe^{3+}. Iron is an essential component for functioning of key enzymes by mediating redox reactions, but in contrast to Fe^{3+}, Ga^{3+} cannot be reduced to the divalent oxidation state (Jakupec and Keppler, 2004; Perabo and Müller, 2007). The approach of interfering with cellular iron metabolism using gallium has been recently applied in treatment of *P. aeruginosa* biofilms (Kaneko *et al.*, 2007). If mature *P. aeruginosa* mushroom-shaped biofilms were exposed to 10 μM $Ga(NO_3)_3$ the interior part of the multicellular structure was killed within 72 hours, whereas cells in the upper area survived the treatment. If mature *P. aeruginosa* mushroom-shaped biofilms were exposed to 100 μM $Ga(NO_3)_3$ the interior part of the multicellular structure was killed within 48 hours. In addition a thin layer of the outermost area of the cap-forming subpopulation was killed (Kaneko *et al.*, 2007). Using *in situ* gene expression analysis, the authors provide evidence, that expression of *pvdA* (encoding for an enzyme required for synthesis of the iron siderophore pyoverdine) is induced in the interior part of the structure, indicating iron-limiting conditions in this area. Therefore killing by gallium of the interior subpopulation is thought to occur by increased uptake of gallium by these cells due to iron starvation and subsequent induced cell death. Gallium was found not to be taken up by the major iron-uptake systems, as pyoverdine as well as pychelin and ferric citrate receptor mutants all exhibit wild type phenotype when grown in the presence of gallium in a flow-chamber setup (Kaneko *et al.*, 2007).

Exposure of biofilms to ciprofloxacin, tetracycline, tobramycin, imipinem, or lysozyme

If *P. aeruginosa* biofilms were exposed either to ciprofloxacin, tetracycline, tobramycin or imipinem, the cell subpopulation situated in the interior area close to the substratum survived the treatment, whereas the cells situated in the upper area of the multicellular structure were killed.

The fluoroquinolone ciprofloxacin has bactericidal effects and induces cell death by interfering with bacterial replication due to inhibition of the DNA gyrase. Ciprofloxacin is administered for treatment of various infections caused by Gram-negative and Gram-positive bacteria. Using *in situ* gene expression analysis, involving a growth activity-dependent fluorescent reporter, it was found that ciprofloxacin specifically targets the biofilm cells exhibiting high metabolic activity in the upper area. By contrast, biofilm cells in the deeper layers exhibiting low metabolic activity survive ciprofloxacin treatment (Pamp *et al.*, submitted). A similar phenotype with respect to the distribution of growth activity and ciprofloxacin-induced cell death was observed for a colony biofilm by *P. aeruginosa* (Walters *et al.*, 2003). The fact that the effect of killing in flow-chamber grown biofilms is found to be restricted to the cells in the upper layer exhibiting high metabolic activity prompted to examine if metabolic activity of the cells in the deeper layers could be increased by e.g. providing an alternative electron acceptor (nitrate) which could subsequently make the cells susceptible to ciprofloxacin. However, when mature *P. aeruginosa* wild type biofilms were exposed simultaneously to ciprofloxacin and nitrate, severe hollowing was induced (Fig. 15a). The hollowing was a result of merging of the cap-forming subpopulations of neighboring mushroom-like structures and dissolution of the stalk. Inside the hollows cells were present, which exhibited increased motility. The cells forming the shell of the hollows were killed to a large degree (Fig. 15a). Another combined treatment, in this case with ciprofloxacin and colistin was found to eradicate almost all cells of the flow-chamber-grown *P. aeruginosa* biofilm (Pamp *et al.*, submitted).

Tetracycline is an antimicrobial agent, which originates from secondary metabolites produced by *Streptomyces* spp. It can inhibit bacterial protein synthesis by preventing attachment of aminoacyl-tRNA to the ribosomal acceptor site (A-site) and thereby induce cell-death. If mature *P. aeruginosa* wild type biofilms were exposed to tetracycline, the cells situated in the upper area of the multicellular structures were killed, whereas the cells situated in the deeper areas survived the treatment (Pamp *et al.*, submitted). An experiment involving treatment of a biofilm formed by a strain, which harbors a growth activity-dependent fluorescent reporter fusion, indicates that tetracycline specifically kills the cells in the upper area, which exhibit high metabolic activity. By contrast, cells in the deeper areas of the biofilm, which exhibit a lower metabolic activity, are not killed by tetracycline (Pamp, unpublished observation). A combined treatment with colistin, which preferably kills the biofilm cells in the interior area, and tetracycline, which preferably kills the biofilm cells in the upper area, enables eradication of almost all cells of the flow-chamber-grown *P. aeruginosa* biofilm (Pamp *et al.*, submitted).

The aminoglycosid tobramycin is a secondary metabolite derived from *Streptomyces* spp. and it can inhibit protein synthesis in Gram-negative bacteria by preventing translocation of peptidyl-tRNA from the A-site to the P-site of the ribosome, and thereby inducing cell-death. If mature *P. aeruginosa* biofilms were exposed to tobramycin, the cells situated in the upper area of the multicellular structures were killed, whereas the cells situated in the deeper areas survived the treatment (Bjarnsholt *et al.*, 2005; Henzer *et al.*, 2003; Kaneko *et al.*, 2007). The efficiency of tobramycin-induced killing in *P. aeruginosa* biofilms was increased by co-administration of furanone C-30, a compound, which was identified to inhibit quorum sensing regulated gene expression (Henzer *et al.*, 2003). Also, a biofilm formed by a mutant strain, which is defective in *las*- and *rhl*-mediated cell-to-cell-communication showed increased sensitivity to tobramycin, indicating a possible role for quorum sensing in tolerance towards tobramycin (Bjarnsholt *et al.*, 2005). Mah and colleagues presented results, which indicate that in biofilm cells of strain PA14 periplasmatic glucans might sequester tobramycin and hence prevent the interaction of

tobramycin with its target. An *ndvB*-mutant, which is deficient in the synthesis of periplasmatic glucans, exhibited increased sensitivity to tobramycin in biofilms (Mah *et al.*, 2003).

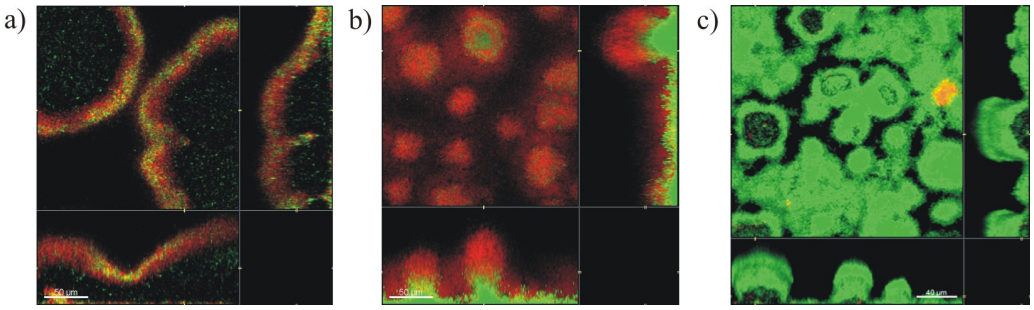

Fig. 15. Antimicrobial treatment of mature *P. aeruginosa* biofilms. *P. aeruginosa* (Gfp) was grown for 4 days on glucose minimal medium in flow-chambers and was then continuously exposed to *a)* 60 μg/ml ciprofloxacin + 7.5 mM nitrate (KNO_3), *b)* 60 μg/ml imipenem, or *c)* 64 μg/ml lysozyme. Images were aquired *a)* 24 hours, *b)* 24 hours and *c)* 13 hours subsequent to the beginning of the treatment. Life cells appear green (Gfp) and dead cells appear red, due to staining with propidium iodide (Pamp, unpubl.).

Imipenem is derived from a secondary metabolite produced in nature by *Streptomyces* spp. It has increased stability towards degradation by β-lactamases compared to e.g. penicillin, and is used for treatment of various infections caused by both Gram-negative and Gram-positive bacteria. As penicillin it interferes with bacterial cell wall synthesis by inhibiting a transpeptidase enzyme that links peptidoglycan molecules, and thereby induces bacterial cell lysis. If mature *P. aeruginosa* biofilms were exposed to 60 μg/ml imipenem (15×MIC) almost all cells in the upper part of the multicellular structures, were killed (lysed), whereas the cells situated in the deeper areas survived the treatment (Fig. 15b). Examination of the upper area of treated biofilms at a higher magnification revealed the presence of large spherical structures, which could be spheroplasts generated by disruption of the cell wall (Pamp, unpublished observation). If mature *P. aeruginosa* biofilms were exposed to subinhibitory concentrations of imipenem (0.5 μg/ml, 1/8×MIC) cells in the upper part of the multicellular structures exhibited increased expression of *ampC* (encoding for a chromosomal encoded β-lactamase), as examined through the use of a fluorescent reporter-fusion (Bagge *et al.*, 2004a). By contrast, cells in the deeper area did not express *ampC*. If mature *P. aeruginosa* biofilms were exposed to 10 μg/ml (2.5×MIC) all cells of the biofilm exhibited expression of *ampC* (Bagge *et al.*, 2004a). Interestingly, exposure of mature *P. aeruginosa* biofilms to subinhibitory concentrations of imipenem for extended periods could increase the biofilm biomass compared to unexposed biofilms. This effect was described to be a consequence of overproduction of the polysaccharide alginate, induced by the low concentrations of imipenem (Bagge *et al.*, 2004b).

Lysozyme is an enzyme, which is present in human fluids (e.g. tears, saliva and respiratory secretions) as well as in cells of the innate immune system (e.g. neutrophils, macrophages). It can degrade bacterial peptidoglycan by hydrolyzing the glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine, leading to cell lysis. In contrast to imipinem, which inhibits peptidoglycan synthesis and induces lysis of the cells, which are situated in the upper layer of the biofilm, treatment of mature *P. aeruginosa* wild type biofilms with lysozyme appears to have a different effect. *P. aeruginosa* biofilms exposed to lysozyme, induced hollowing in the interior area of the biofilm, which is situated closest to the substratum (Fig. 15c). Inside the hollows single cells were observed, which exhibited extensive motility. In addition, a few single dead cells were present. The remaining cells of the multicellular structure were seemingly unaffected (Pamp, unpublished observation).

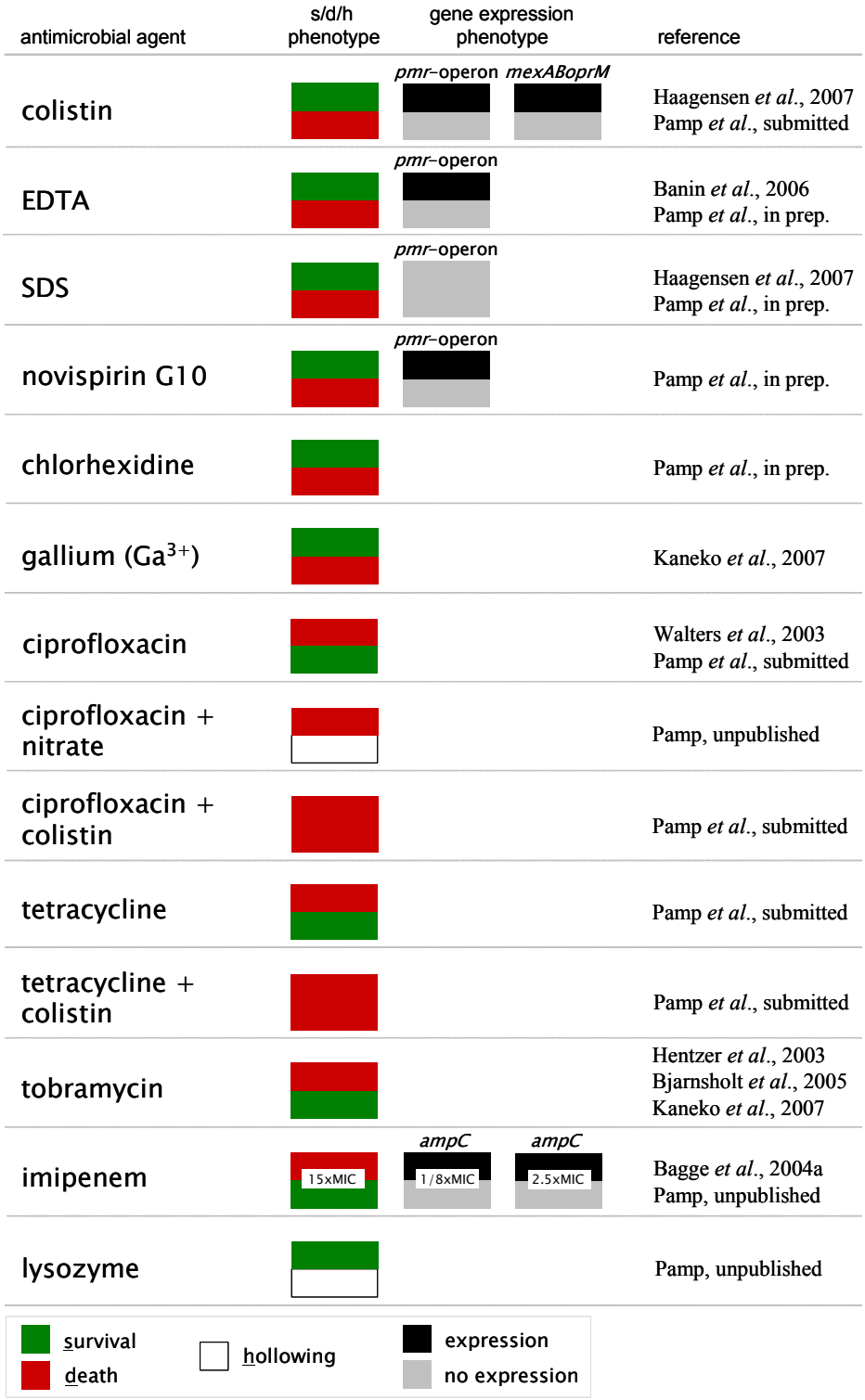

Fig. 16. Antimicrobial tolerance & susceptibility phenotype of mature *P. aeruginosa* biofilms presented schematically as color-coded graphical fingerprint. For explanations regarding the representation please see the information provided in the figure legend to figure 13. *Red*: most cells in this area exhibit sensitivity towards the antimicrobial compound. *Green*: most cells in this area survive the treatment of the antimicrobial compound. *White*: the 'hollowing' pattern is induced in this area. *Black*: the gene/operon is expressed in cells present in this area. *Grey*: the gene is not (or marginal) expressed in cells present in this area. The representation here is a simplification of the described observations from the various studies and should be regarded as such.

One explanation for the different effects of imipinem and lysozyme on *P. aeruginosa* biofilms could be, that imipinem might exert its effects preferentially on proliferating cells, whereas the effect of lysozyme might not be dependent on cell proliferation.

It is striking that upon exposure of *P. aeruginosa* biofilms, surviving and dead cells are not randomly distributed. Instead, the effect of antimicrobial compounds is confined to one of the two physiological distinct subpopulations, which have been identified to be prevailing in *P. aeruginosa* biofilms (see chapter 3.1). The observations suggest, that the effect of antimicrobial agents, which interfere with basic cellular metabolic processes, such as replication, transcription, translation, or peptidoglycan synthesis, appears to a major part to depend on the metabolic/physiological activity of the prevailing biofilm cells. Therefore, the efficiency of a conventional antimicrobial compound to induce cell death in a population of biofilm cells might depend on the number of biofilm cells, which reflect the active state of a particular target, such as those cells undergoing replication will be more vulnerable to compounds interfering with replication processes than cells that do not replicate. This is in agreement with observations showing that antimicrobial agents, which interfere with peptidoglycan synthesis, replication or translation, effectively induce cell death in exponential phase cells, whereas their efficiency is relatively low on stationary phase cells (Spoering and Lewis, 2001). In comparison, biofilm populations of cells exhibited an overall median sensitivity to these compounds (Spoering and Lewis, 2001), which is in agreement with observations showing that biofilm cell populations consists of a mixture of both, cells exhibiting high and cells exhibiting low metabolic/physiological activity (e.g. Pamp *et al.*, submitted; Rani *et al.*, 2007; Sternberg *et al.*, 1999; Wentland *et al.*, 1996; Werner *et al.*, 2004; Haagensen, personal communication). Other factors such as the inherent characteristics of a particular antimicrobial agent (e.g. hydrophobicity) and inherent characteristics of biofilms (e.g. matrix synthesis), might impact on the rate of diffusion of a particular compound through the multicellular structure as well. In addition, some cells might reflect a certain cell type in which they are inherently invulnerable towards a particular antimicrobial agent. The reason why membrane-targeting compounds and gallium preferentially kill cells in the deeper areas of the biofilm is unknown at present. Also the factors, which confer tolerance to the cells in the upper layer of the biofilm towards these compounds, are not entirely uncovered. Regarding tolerance development to colistin it appears that the metabolic active cells are able to adapt to colistin by LPS-modification, mediated by the *pmr*-operon and antimicrobial efflux, mediated by *mexAB-oprM*. However, at least with respect to *pmr*-mediated LPS-modification it appears that this mechanism does not confer tolerance to the membrane-targeting compounds EDTA and SDS. It will be interesting to investigate, if one common determining factor is responsible for the spatial distribution of live and dead cells upon exposure with membrane-targeting compounds and gallium, or if various mechanisms specific for each antimicrobial agent exist.

4 Concluding remarks

The majority of microbial cells on Earth live in intimate relationship with other microbial cells to jointly form communities, also called biofilms. Biofilms exist in a great variety of shapes, sizes and composition and most of the microbial communities are associated with animals, plants, and abiotic matter. One challenge among microbiologist is to unravel possible common themes among microbial communities, and to learn about interactions taking place between the participating biofilm cells and/or between the biofilm cells and their environment.

Studies involving laboratory-grown biofilms have provided intriguing insight into the fundamental capacities of microorganisms to assemble into multicellular communities. Model systems such as flow-chamber-grown biofilms and colony biofilms, reveal a set of inherent elements of microorganisms that can facilitate their organization into multicellular communities. Among those elements can be e.g. the production of matrix compounds, the secretion of surfactants, cell-surface bound proteins, cellular migration, and regulatory elements such as signal transduction systems, and intra- and extra-cellular signal messenger molecules. In this experimental thesis work biosurfactants, flagella-driven motility and chemotaxis signal transduction were found to be elements involved in certain steps of the organization of cells into multicellular communities by the model-organism *P. aeruginosa*. A number of studies, including this thesis work, indicate that cells differentiate into different cell types during the process of development of a multicellular structure. These cell types express a phenotype, which distinguishes them from other cells and suggest that the different cell types exert different functions during biofilm development. A common theme seems to be that cells of the same type spatially co-localize and take up similar positions in space, suggesting that environmental factors impact on the differentiation process. Mature multicellular structures reveal a spatial organization that can reflect the developmental process of assembly. Confined subpopulations can be distinguished with respect to their ability to migrate, their metabolic/physiological state, gene expression, productivity of extracellular (macro-) molecules, responsiveness to various environmental stimuli and sensitivity to toxic compounds. These insights are supported by various observations from this experimental thesis work on *P. aeruginosa* biofilms, such as the observations that i) secreted biosurfactants facilitate migration of cells to form cap-subpopulations, ii) the cap-forming cell subpopulation is susceptible to ciprofloxacin, tetracycline and imipinem, and iii) expression of the *pmr*-operon and *mexAB-oprM* in metabolic active cells confers tolerance to colistin.

Future studies, facilitated by sophisticated approaches and new technologies, will increase the understanding of the microbial life in multicellular communities. Research studies with focus at a lower level of organization will be able to uncover expression profiles of single cells within the community and reveal dependencies between single members of a multicellular community. Research studies with focus at a higher level of organization will be able to uncover the actual species diversity within a microbial community in a given ecological niche in nature, and reveal dependencies between the various species within a community and dependencies between a microbial community and its environment.

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Paper 1

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Multiple Roles of Biosurfactants in Structural Biofilm Development by *Pseudomonas aeruginosa*[▽]

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Recent studies have indicated that biosurfactants produced by *Pseudomonas aeruginosa* play a role both in maintaining channels between multicellular structures in biofilms and in dispersal of cells from biofilms. Through the use of flow cell technology and enhanced confocal laser scanning microscopy, we have obtained results which suggest that the biosurfactants produced by *P. aeruginosa* play additional roles in structural biofilm development. We present genetic evidence that during biofilm development by *P. aeruginosa*, biosurfactants promote microcolony formation in the initial phase and facilitate migration-dependent structural development in the later phase. *P. aeruginosa rhlA* mutants, deficient in synthesis of biosurfactants, were not capable of forming microcolonies in the initial phase of biofilm formation. Experiments involving two-color-coded mixed-strain biofilms showed that *P. aeruginosa rhlA* mutants were defective in migration-dependent development of mushroom-shaped multicellular structures in the later phase of biofilm formation. Experiments involving three-color-coded mixed-strain *P. aeruginosa* biofilms demonstrated that the wild-type and *rhlA* and *pilA* mutant strains formed distinct subpopulations on top of each other dependent on their ability to migrate and produce biosurfactants.

Evidence is accumulating that many infections display elevated tolerance towards antimicrobial attack because the causative bacteria reside in biofilms (8, 15, 51). The opportunistic human pathogen *Pseudomonas aeruginosa* has become a model organism for studying biofilm development. When *P. aeruginosa* is grown as a biofilm in flow chambers, it often develops mushroom-shaped multicellular structures separated by liquid-filled channels (9, 26, 32). The cap-forming subpopulation and the stalk-forming subpopulation of these mushroom-shaped structures in many cases display differential tolerance to antimicrobial compounds. For example, the antibiotic tobramycin was shown to kill preferentially bacteria in the cap portion of the mushroom-shaped structures, whereas the antibiotic colistin, the detergent sodium dodecyl sulfate, and the chelator EDTA were shown to kill preferentially bacteria in the stalk portion of the mushroom-shaped structures (3, 5, 19, 21). Knowledge about subpopulation development in biofilms, and the way the subpopulations interact and change during structural biofilm development, may be useful for creating strategies to control biofilm formation and eradicate persistent infections.

Studies of liquid flow and molecular diffusion in flow chamber-grown biofilms have led to the proposal that the channels and interstitial voids between the microcolonies may function as a circulation system for efficient nutrient supply and waste product removal (11, 52). Biosurfactants produced by *P. aeruginosa* via the RhlA gene product were shown to be important for maintenance of the water channels between the mushroom-shaped multicellular structures in biofilms (10). A study which employed fluorescent reporter genes indicated that biosurfactant synthesis preferentially takes place in microcolonies during early stages of biofilm development and in the stalk portion of the mushroom-shaped structures in more mature *P. aeruginosa* biofilms (33). In addition, evidence has been presented that the biosurfactants produced by *P. aeruginosa* are involved in the dispersion of cells from biofilms (5, 24, 47).

P. aeruginosa produces a number of biosurfactants, of which the three most abundant are 3-(3-hydroxyalkanoyloxy)alcanoic acid (HAA), L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (mono-rhamnolipid), and L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (di-rhamnolipid) (13, 14, 44, 46). HAA is synthesized via the RhlA enzyme and is converted to mono-rhamnolipid by the RhlB enzyme (14, 39). Mono-rhamnolipid is converted to di-rhamnolipid by the RhlC enzyme (44). The *rlhAB* operon and *rhlC* gene are induced by acyl homoserine lactone-activated RhlR and are thus under quorum-sensing control (39–42, 44). In addition, phosphate limitation and the presence of nitrate have been shown to promote the synthesis of rhamnolipids, while ammonium and high amounts of iron have been shown to repress the production of rhamnolipids (18, 37, 38).

The formation of the mushroom-shaped multicellular structures in *P. aeruginosa* biofilms under some conditions occurs in a sequential process which involves a nonmotile bacterial subpopulation that forms the mushroom stalks by clonal cell proliferation in certain foci on the substratum and a migrating bacterial subpopulation that forms the mushroom caps via a process which requires type IV pili (26). Type IV pili have been implicated in two kinds of surface-associated motility in *P. aeruginosa*. One form of surface-associated translocation of *P. aeruginosa* which requires functional flagella and biosurfactant production, and under some conditions type IV pili, has been termed swarm-

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TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristics or sequence	Source and/or reference
P. aeruginosa strains		
PAO1	Wild type	23; obtained from J. S. Mattick, University of Queensland Brisbane
PAO1 Gfp	PAO1 tagged with EGFP in a mini-Tn*7* construct; Gm^r	27
PAO1 Yfp	PAO1 tagged with EYFP in a mini-Tn*7* construct; Gm^r	27
PAO1 *rhlA*	*rhlA*::Gm^r	44; kindly provided by G. O'Toole, Dartmouth Medical School
PAO1 *rhlA* Yfp	*rhlA* inactivated by transferring *rhlA*::Gm^r from strain PAO1 *rhlA* via transduction into PAO1 wild type; tagged with EYFP in a mini-Tn*7* construct; Gm^r Sm^r	This study
PAO1 *rhlA* Cfp	*rhlA* inactivated by transferring *rhlA*::Gm^r from strain PAO1 *rhlA* via transduction into PAO1 wild type; tagged with ECFP in a mini-Tn*7* construct; Gm^r Sm^r	This study
PAO1 *pilA*	*pilA* inactivated by allelic displacement with a tellurite resistance cassette using pTTN80; Tel^r	27
PAO1 *pilA* Cfp	PAO1 *pilA* tagged with CFP; Tel^r Sm^r	This study
PAO1 *pilArhlA* Cfp	*pilA* inactivated by allelic displacement in strain PAO1 *rhlA* CFP using pTTN80; Tel^r Gm^r Sm^r	This study
E. coli HB101	*recA thi pro leu hsdRM*; Sm^r; strain used for maintenance and proliferation of pTTN80 and the plasmids used for fluorescent tagging	25
Plasmids		
pTTN80	pCK318 with *pilA*::Tel^r cassette cloned in the NotI site; Ap^r Tel^r; used for allelic displacement	27
pUX-BF13	*mob*$^+$ *ori*-R6K; Ap^r; helper plasmid providing the Tn*7* transposition functions in *trans*	4
pBK-mini-Tn*7*(Km, Sm)$P_{A1/04/03}$-eyfp-a	*mob*$^+$; Ap^r Sm^r Km^r; delivery plasmid for mini-Tn*7*(Km, Sm)$P_{A1/04/03}$-eyfp	31
pBK-mini-Tn*7*(Km, Sm)$P_{A1/04/03}$-ecfp-a	*mob*$^+$; Ap^r Sm^r Km^r; delivery plasmid for mini-Tn*7*(Km, Sm)$P_{A1/04/03}$-ecfp	31
pEX1.8	pEX1 carrying ori (*P. aeruginosa*) as 1.8-kb PstI fragment from pRO1614 in StyI site	41
pEX1.8-rhlAB	pEX1.8 carrying *rhlAB* including the *rhlA* promoter	5
Primers		
Tn*7*-GlmS	5′-AATCTGGCCAAGTCGGTGAC-3′	
Tn*7*-R109	5′-CAGCATAACTGGACTGATTTCAG-3′	
rhlA-fw	5′-CCGCTGAGTTACTTGTCTGC-3′	
rhlA-rev	5′-TGCGTTATGCAACCGCAAAG-3′	
pilA-fw	5′-GAAGTACGCGGTCACCTG-3′	
pilA-rev	5′-CGGAGATGCCTACAAAGAGC-3′	

ing motility (29, 45, 49). Another form of surface-associated translocation of *P. aeruginosa*, which requires type IV pili, has been termed twitching motility (20, 34, 48). Presently, it is not known whether the type IV pili-driven motility that plays a role in mushroom cap formation in *P. aeruginosa* biofilms should be regarded as twitching, swarming, or another kind of surface-associated motility.

Because surface-associated motility and biosurfactant production evidently both have roles in structural biofilm formation by *P. aeruginosa*, we found it of interest to investigate whether the biosurfactants produced by *P. aeruginosa* might play a role in structural biofilm development by facilitating surface-associated motility. We present evidence that migration-dependent formation of the cap portion of the mushroom-shaped structures in *P. aeruginosa* biofilms is facilitated by biosurfactant production. In addition, we present evidence that biosurfactant production is necessary for initial microcolony formation in *P. aeruginosa* biofilms.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured in Luria broth (LB) medium at 37°C. *P. aeruginosa* strains were cultured in LB medium at 37°C during the procedure of genetic manipulation. In motility plate assays, orcinol assays, and for cultivation of biofilms, AB minimal medium supplemented with glucose as indicated was used. AB medium consists of $(NH_4)_2SO_4$ (15.1 mM), $Na_2HPO_4 \cdot 2H_2O$ (33.7 mM), KH_2PO_4 (22.0 mM), NaCl (0.051 M), $MgCl_2$ (1 mM), $CaCl_2$ (0.1 mM), and trace metals (100 μl/liter). The trace metal solution contained $CaSO_4 \cdot 2H_2O$ (200 mg/liter), $FeSO_4 \cdot 7H_2O$ (200 mg/liter), $MnSO_4 \cdot H_2O$ (20 mg/liter), $CuSO_4 \cdot 5H_2O$ (20 mg/liter), $ZnSO_4 \cdot 7H_2O$ (20 mg/liter), $CoSO_4 \cdot 7H_2O$ (10 mg/liter), $NaMoO_4 \cdot H_2O$, and H_3BO_3 (5 mg/liter). Antimicrobial agents were used where appropriate at the following concentrations: for *E. coli*, ampicillin (Vepidan ApS, Denmark) at 100 μg/ml; for *P. aeruginosa*, gentamicin sulfate (Biochrome AG, Germany) at 30 μg/ml, streptomycin sulfate (Sigma) at 300 μg/ml, potassium tellurite (Sigma) at 150 μg/ml, and carbenicillin (Sigma) at 200 μg/ml. The *P. aeruginosa* PAO1 (23) strain from John Matticks' laboratory and an isogenic *pilA*::Tel^r derivative (27) were used in this study. The *rhlA* mutant strain was obtained by transferring the mutated *rhlA* gene from *P. aeruginosa* PAO1 *rhlA*::Gm^r (44) (kindly provided by G. A. O'Toole) into *P. aeruginosa* PAO1, using the transducing phage E79*tv*2

(36). The *P. aeruginosa* PAO1 *pilA rhlA* double mutant strain was derived from the *rhlA* mutant by allelic displacement of *pilA* with *pilA*::Tel^r using the knockout plasmid pTTN80. The strains were fluorescently tagged at an intergenic neutral chromosomal locus downstream of the *glmS* gene with *ecfp*, *eyfp*, or *egfp* in mini-Tn*7* constructs as described previously (31). Plasmids were transformed into *P. aeruginosa* strains using electroporation (25 μF, 200 Ω, <5 ms, 2.5 kV).

Rhamnolipid assay. The concentration of rhamnolipids in culture supernatants was determined by the orcinol method as previously described (5, 28, 47), with modifications. Briefly, *P. aeruginosa* strains were grown at 30°C for 4 days in AB minimal medium supplemented with 10 mM glucose. A 0.5-ml aliquot of culture supernatant was extracted twice with 2 volumes of diethyl ether (high-performance liquid chromatography grade; Sigma). The ether fractions were pooled, evaporated to dryness, and reconstituted in 0.25 ml distilled H_2O. A 100-μl aliquot of each sample was diluted 1:10 in orcinol reagent and heated at 80°C for 30 min. Orcinol reagent was prepared immediately prior to use and consisted of 7.5 volumes of 60% (vol/vol) sulfuric acid and 1 volume of 1.6% (wt/vol) orcinol in distilled water. After heating, the samples were allowed to cool at room temperature for 15 min, and absorbance (A_{421}) was measured and compared with rhamnose standards. The concentration of rhamnolipids was determined by the relation that 1.0 mg of rhamnose corresponds to 2.5 mg of rhamnolipid (41).

Twitching motility plate assay. Twitching motility was assayed on plates composed of AB minimal medium supplemented with 10 mM glucose and solidified with 1.0% agar. The plates were dried overnight at room temperature and point inoculated through the agar surface with 2.5-μl aliquots taken from overnight cultures of *P. aeruginosa*. Overnight cultures of *P. aeruginosa* were grown in AB minimal medium, supplemented with 30 mM glucose, at 30°C under vigorous shaking. All plates were incubated at 30°C for 48 h. Twitching motility was visualized by staining with Coomassie brilliant blue R250 (Sigma) as described by Semmler et al. (48). To complement the twitching motility-deficient phenotype of the *rhlA* mutant, 0.0005% Tween 20 was added to the agar medium where indicated. Twitching motility of *P. aeruginosa rhlA* containing either pEX1.8 or pEX1.8-*rhlAB* was examined in the presence of carbenicillin.

Cultivation of biofilms. Biofilms were cultivated in three-channel flow cells with individual channel dimensions of 1 by 4 by 40 mm and covered with a glass coverslip serving as substratum for biofilm formation. The flow system was assembled and prepared as described elsewhere (50). AB minimal medium supplemented with 0.3 mM glucose as carbon source was used as growth medium. Individual flow chambers were inoculated with 300-μl aliquots taken from overnight cultures of *P. aeruginosa*, adjusted to an optical density at 500 nm of 0.005. Overnight cultures of *P. aeruginosa* strains were grown in AB minimal medium supplemented with 30 mM glucose at 30°C under vigorous shaking. *P. aeruginosa rhlA* strains containing either pEX1.8 or pEX1.8-*rhlAB* were grown as overnight cultures in the presence of carbenicillin. After inoculation, flow chambers were left without flow for 1 h to allow bacterial attachment. The flow system was incubated at 30°C, and a laminar flow with a mean flow velocity of 0.2 mm s^{-1} was achieved using a Watson Marlow 205S peristaltic pump. *P. aeruginosa rhlA* strains harboring either pEX1.8 or pEX1.8-*rhlAB* were cultivated as biofilms without supplementation of carbenicillin. Loss of plasmid during 4 days of growth in biofilms was not observed as examined by plating of cells derived from biofilms on LB agar medium with and without carbenicillin.

Microscopy and image processing. Image acquisition was performed by the use of a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany) equipped with an argon laser and with detectors and filter sets for simultaneous monitoring of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). Spectral imaging of mixed three-color-coded biofilms (CFP, green fluorescent protein [GFP], and YFP) was carried out using the Zeiss META module. Lambda stacks within a spectral range from 453.5 to 549.8 nm were recorded using laser excitation at 458, 488, and 514 nm. Reference emission spectra from single-color-coded biofilms were used for linear unmixing of the mixed color-coded biofilms. Images were obtained using a 40×/1.3 Plan-Neofluar oil objective. Simulated three-dimensional images, shadow projections, and vertical cross-sections were generated using the Imaris software package (Bitplane AG).

RESULTS

In order to examine the effect of biosurfactant production on biofilm development by *P. aeruginosa*, we constructed isogenic *P. aeruginosa* strains with and without the capability to produce biosurfactants. We inactivated the *rhlA* gene in our *P. aeruginosa* PAO1 strain by the use of transduction with phages that had been propagated on a *P. aeruginosa rhlA*::Gm^r strain. Insertion of the *rhlA*::Gm^r cassette in our PAO1 strain was confirmed by PCR, and deficiency of biosurfactant production was confirmed by a drop-collapsing assay (data not shown). In addition, an orcinol assay showed that the wild-type PAO1 produced 50.9 μg rhamnolipid/ml ($n = 3$; standard deviation [SD], 7.1) when it was grown in the medium used to cultivate biofilms, compared to 0.58 μg rhamnolipid/ml ($n = 3$; SD, 1.1) measured in cultures of the *rhlA* mutant. As biosurfactants have been described to facilitate swarming motility (25), we examined the ability of the PAO1 *rhlA* mutant and wild type to swarm on semisolid glucose minimal medium and found that the *rhlA* mutant was deficient in swarming, as expected (data not shown). Subsequently, we tested the ability of the *rhlA* mutant to perform twitching motility and found that the rate of expansion at the interstitial phase between a plastic surface and agar was significantly reduced for the *rhlA* mutant compared to the wild type (Fig. 1A and Table 2), suggesting that biosurfactants can also facilitate twitching motility. The *P. aeruginosa pilA* mutant (which does not produce type IV pili) was included as a nontwitching control (Fig. 1A). The twitching defect of the *rhlA* mutant was complemented in *P. aeruginosa* PAO1 *rhlA*(pEX1.8-*rhlAB*), which contains a plasmid-

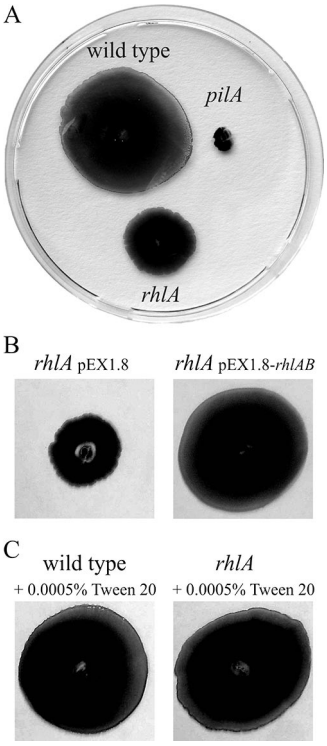

FIG. 1. Twitching motility plate assays. Glucose minimal medium agar plates were point inoculated with *P. aeruginosa* PAO1 strains and incubated at 30°C for 48 h. The twitching zones were visualized by staining with Coomassie brilliant blue after incubation. (A) Twitching zones of *P. aeruginosa* PAO1 Yfp wild type, *P. aeruginosa* PAO1 *pilA*::Tel^r (Cfp), and *P. aeruginosa* PAO1 *rhlA*::Gm^r Yfp. (B) Twitching zones of *P. aeruginosa* PAO1 *rhlA*::Gm^r Yfp harboring either the vector control plasmid pEX1.8 or the plasmid pEX1.8-*rhlAB* expressing *rhlAB* in *trans*. (C) Twitching zones of *P. aeruginosa* PAO1 Yfp wild type and *P. aeruginosa* PAO1 *rhlA*::Gm^r Yfp on medium containing 0.0005% Tween 20.

TABLE 2. Quantification of twitching motility

P. aeruginosa strain and supplementation	Median diam[a] (cm) ± SD
P. aeruginosa PAO1 Yfp	3.25 ± 0.12
P. aeruginosa PAO1 *rhlA* Yfp	1.60 ± 0.11
P. aeruginosa PAO1 *rhlA* Yfp pEX1.8	1.60 ± 0.21
P. aeruginosa PAO1 *rhlA* Yfp pEX1.8-*rhlAB*	2.85 ± 0.16
P. aeruginosa PAO1 Yfp + 0.0005% Tween 20	3.30 ± 0.19
P. aeruginosa PAO1 *rhlA* Yfp + 0.0005% Tween 20	3.15 ± 0.21

[a] Based on 10 replicates for each strain/supplement combination.

borne *rhlAB* operon (Fig. 1B and Table 2). To further substantiate that surfactants can facilitate twitching motility, we investigated if the presence of the surfactant Tween 20 could restore twitching motility of the *rhlA* mutant in the plate assay. In the presence of very low concentrations of Tween 20, the *rhlA* mutant was able to migrate by twitching motility at the plastic-agar interstitial surface to the same extent as the wild type (with or without Tween 20) (Fig. 1C and Table 2).

We have previously found that when *P. aeruginosa* was grown in flow chambers with glucose as carbon source, the bacteria differentiated into a nonmotile and a motile subpopulation and formed mushroom-shaped multicellular structures with the nonmotile subpopulation in the stalk portion and the motile subpopulation eventually settling in the cap portion (26). When a biofilm was initiated with a mixture of *P. aeruginosa* wild type and *P. aeruginosa pilA* mutant, the nonmotile *pilA* mutant cells formed mushroom stalks only, whereas the motile wild-type bacteria were able to form mushroom caps on top of the mushroom stalks formed by the *pilA* mutants (26). To address whether biosurfactants may facilitate cap formation by the motile subpopulation in *P. aeruginosa* biofilms, we inoculated glucose minimal medium-perfused flow chambers with 1:1 mixtures of CFP-tagged *pilA* mutant and either YFP-tagged wild type or YFP-tagged *rhlA* mutant and investigated structural biofilm development in the flow chambers by the use of confocal laser scanning microscopy (CLSM). After 4 days, the wild type had formed cap-shaped structures on top of the stalk-shaped structures formed by the *pilA* mutant (Fig. 2A), as has been shown previously by Klausen et al. (26). By day 4 the *rhlA* mutant also had formed cap-shaped structures on top of the stalk-forming *pilA* mutant, but the caps formed by the *rhlA* mutant were significantly smaller than those formed by the wild type (Fig. 2). The defect of the *rhlA* mutant in cap structure formation could be complemented by introducing the plasmid pEX1.8-*rhlAB*, expressing the *rhlAB* operon in *trans*. The *rhlA*(pEX1.8-*rhlAB*) strain formed caps on top of the stalk-forming *pilA* mutant which were similar to those formed by the wild type, whereas the *rhlA*(pEX1.8) strain formed caps similar to those formed by the *rhlA* mutant (Fig. 2). Analysis of 50 randomly chosen mushroom-shaped structures in each of

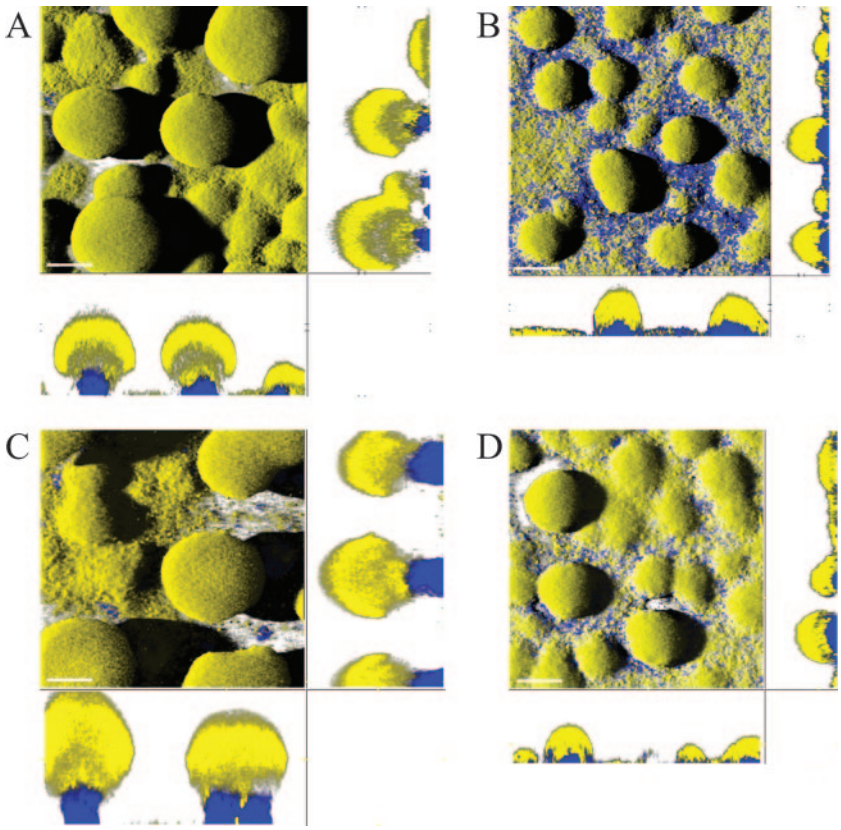

FIG. 2. Confocal laser scanning micrographs of 4-day-old biofilms, initiated with a 1:1 mixture of either *P. aeruginosa pilA*::Tel^r Cfp and *P. aeruginosa* Yfp wild type (A), *P. aeruginosa pilA*::Tel^r Cfp and *P. aeruginosa* PAO1 *rhlA*::Gm^r Yfp (B), *P. aeruginosa pilA*::Tel^r Cfp and *P. aeruginosa* PAO1 *rhlA*::Gm^r Yfp(pEX1.8-*rhlAB*) (C), or *P. aeruginosa pilA*::Tel^r Cfp and *P. aeruginosa* PAO1 *rhlA*::Gm^r Yfp(pEX1.8) (D). The images show top-down shadow projections (230 μm by 230 μm) with two flanking images representing sections in the *x-z* and the *y-z* planes. Bar, 40 μm.

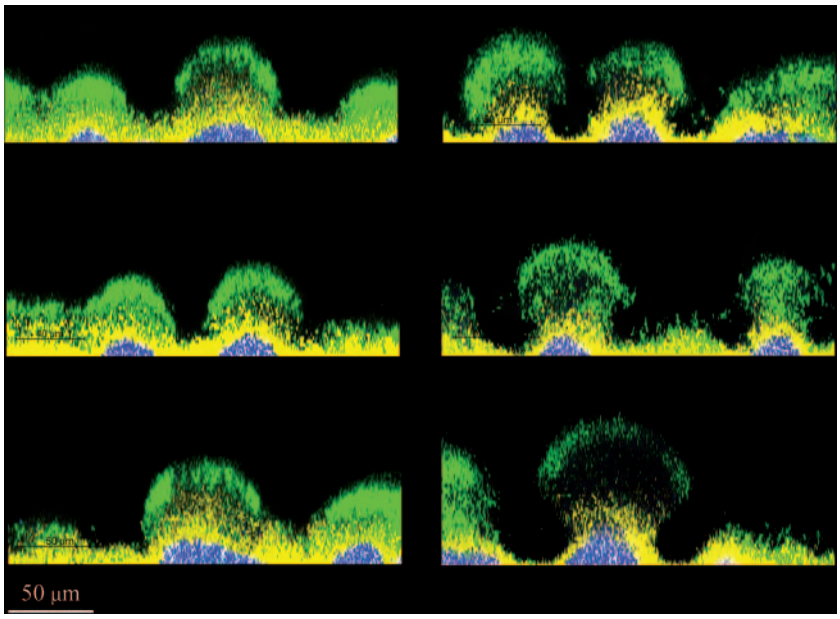

FIG. 3. Confocal laser scanning micrographs of biofilms initiated with a 1:1:1 mixture of *P. aeruginosa pilA*::Tel^r Cfp, *P. aeruginosa rhlA*::Gm^r Yfp, and *P. aeruginosa* PAO1 Gfp wild type. Representative vertical sections acquired at three different locations in 3- (left) or 4-day-old (right) biofilms are shown.

the 4-day-old mixed-strain biofilms showed that the wild type formed caps with an average height of 65.34 μm (SD, 9.80), while the *rhlA* mutant formed caps with an average height of 31.30 μm (SD, 5.99). The complemented strain *rhlA*(pEX1.8-*rhlAB*) formed caps with an average height of 65.71 μm (SD, 10.37), whereas the vector control strain *rhlA*(pEX1.8) formed caps with an average height of 27.02 μm (SD, 5.28). The experiment therefore suggested that biosurfactants produced by *P. aeruginosa* have a role in facilitating mushroom cap formation.

The experiments indicating that biosurfactants may facilitate cap formation by facilitating bacterial migration also imply that a putative effect of the biosurfactants to a high degree is confined to the biosurfactant-producing subpopulation, as the stalk-forming *pilA* subpopulation in the experiments described above was capable of synthesizing biosurfactants. If biosurfactant production (i) facilitates colonization of the upper part of *P. aeruginosa* biofilms by facilitating bacterial migration, and (ii) exerts an effect which to a large extent is confined to the biosurfactant-producing subpopulation, then a mixed biofilm formed by *P. aeruginosa pilA*, *rhlA*, and wild-type strains should form multicellular structures with the *pilA* mutant at the base, the *rhlA* mutant on top of the *pilA* mutant, and the wild type on top of the *rhlA* mutant. To investigate this hypothesis we inoculated flow chambers with a 1:1:1 mixture of CFP-tagged *pilA* mutant, YFP-tagged *rhlA* mutant, and GFP-tagged wild type. As shown in Fig. 3, in support of the hypothesis, (i) the *pilA* mutants, which can produce biosurfactants but are unable to migrate, formed microcolonies at the substratum, (ii) the *rhlA* mutants, which cannot produce biosurfactants and in a twitching motility assay showed reduced ability to migrate, were able to colonize on top of the microcolonies formed by the *pilA* mutants, and (iii) the wild-type bacteria, which are capable of producing biosurfactants and have no migration deficiency, were able to colonize on top of the *rhlA* mutants. Because the fluorescence emission peaks of CFP, GFP, and YFP are close to each other, simultaneous detection of these three fluorescent proteins is not possible by the use of conventional CLSM. Therefore, simultaneous detection of the YFP-, GFP-, or CFP-tagged cells in the biofilm was carried out by the use of a special detector system mounted on the microscope (as described in Materials and Methods) which, as apparent in Fig. 3, has diminished signal detection compared to conventional CLSM.

Although the effect of the biosurfactants apparently to a high degree is confined to the biosurfactant-producing subpopulation, we could not exclude that biosurfactants produced by the *pilA* or wild-type subpopulations in the mixed-strain biofilms to some extent could facilitate migration of the *rhlA* mutant. If biosurfactants were necessary for the motile subpopulation to migrate on the *pilA* microcolonies, we would expect that the *rhlA* mutant would not be able to colonize *pilA* microcolonies which are not capable of providing biosurfactants. To address this, we knocked out the *pilA* gene in the *rhlA* mutant by the use of allelic exchange, inoculated a flow cell with a 1:1 mixture of CFP-tagged *pilA rhlA* double mutant and YFP-tagged *rhlA* mutant, and investigated biofilm development in glucose minimal medium. We expected that the *pilA rhlA* double mutant would form mushroom stalks and that we would be able to investigate the ability of the *rhlA* mutant to form mushroom caps in a biofilm entirely devoid of biosurfactants. Surprisingly, however, microcolonies did not develop in the biofilm containing the mixture of the *pilA rhlA* double mutant and the *rhlA* mutant. Instead, the bacteria formed a flat biofilm, as shown in Fig. 4, which depicts a CLSM micrograph acquired in a 4-day-old *pilA rhlA-rhlA* mixed biofilm.

Because our experiments with mixed-strain *P. aeruginosa* biofilms surprisingly had indicated that biosurfactants, in addition to their role in the formation of mature biofilm struc-

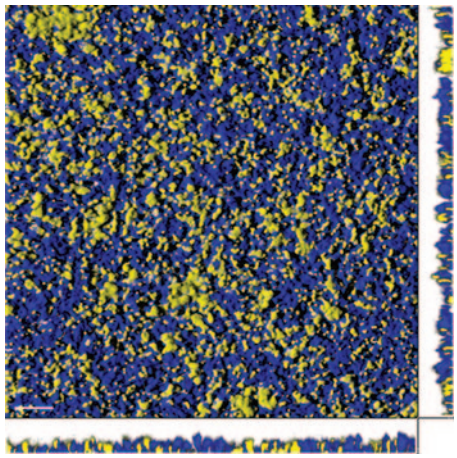

FIG. 4. Confocal laser scanning micrograph of a 4-day-old biofilm, initiated with a 1:1 mixture of *P. aeruginosa rhlA*::Gm^r Yfp and *P. aeruginosa pilA*::Tel^r *rhlA*::Gm^r Cfp. The image shows a top-down shadow projection (230 μm by 230 μm) with two flanking images representing sections in the *x-z* and the *y-z* planes. Bar, 20 μm.

tures, might also play a role in the formation of the initial microcolonies, we found it of interest to examine the role of biosurfactant production on biofilm development in more simple model systems consisting of mono-strain *P. aeruginosa* biofilms. While the wild type and the *pilA* mutant after 4 days of development had formed a biofilm with mushroom-shaped structures (Fig. 5A) and irregular protruding structures (Fig. 5C), respectively, the *rhlA* mutant and the *pilA rhlA* double mutant both formed flat biofilms (Fig. 5B and D), supporting the hypothesis that biosurfactants are necessary for initial microcolony formation.

To examine further the role of biosurfactants in initial microcolony formation, we examined young *P. aeruginosa* biofilms. After 16 h of development in glucose minimal medium-perfused flow chambers, the wild type had formed small microcolonies (Fig. 6A), whereas the *rhlA* mutant had formed a flat thin biofilm (Fig. 6B). When the *rhlA* mutant and the wild type were present in the flow chamber in a 1:1 mixture, the *rhlA* mutant was able to form initial microcolonies of the same size as those formed by the wild type (Fig. 6C), suggesting that biosurfactant produced by the wild type enabled microcolony formation by the *rhlA* mutant.

DISCUSSION

Because previous studies had indicated that surface-associated motility and biosurfactant production both play roles in structure formation in *P. aeruginosa* biofilms (10, 26), we found it of interest to investigate if the role of biosurfactants in structural *P. aeruginosa* biofilm formation can be attributed to

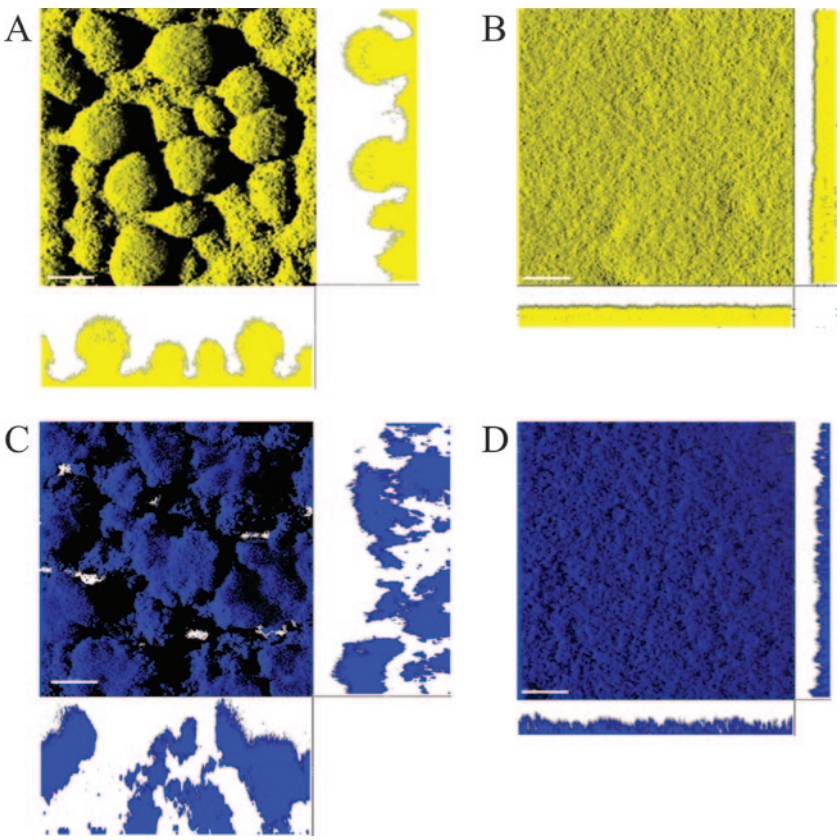

FIG. 5. Confocal laser scanning micrographs of 4-day-old biofilms formed by *P. aeruginosa* PAO1 Yfp wild type (A), *P. aeruginosa rhlA*::Gm^r Yfp (B), *P. aeruginosa pilA*::Tel^r Cfp (C), and *P. aeruginosa pilA*::Tel^r *rhlA*::Gm^r Cfp (D). The images show top-down shadow projections (230 μm by 230 μm) with two flanking images representing sections in the *x-z* and the *y-z* planes. Bar, 40 μm.

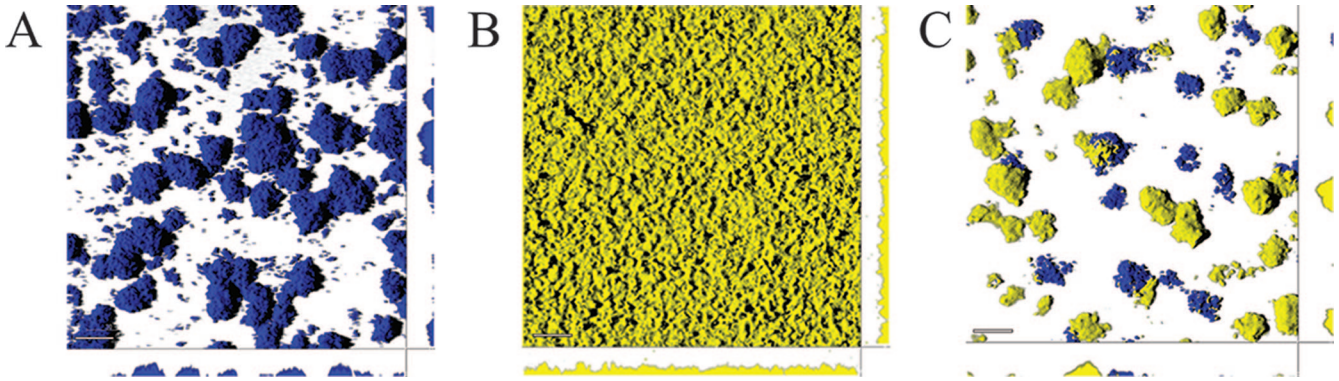

FIG. 6. Confocal laser scanning micrographs of 16-h-old biofilms formed by *P. aeruginosa* PAO1 Cfp wild type (A), *P. aeruginosa rhlA*::Gm^r Yfp (B), and a 1:1 mixture of *P. aeruginosa* PAO1 Cfp wild type and *P. aeruginosa rhlA*::Gm^r Yfp (C). The images show top-down shadow projections (230 μm by 230 μm) with two flanking images representing sections in the *x-z* and the *y-z* planes. Bar, 30 μm.

facilitation of bacterial migration. As discussed below, our results indicate that biosurfactants produced by *P. aeruginosa* indeed do play roles in migration-mediated structure formation in the later stages of biofilm development and, in addition, have an impact on microcolony formation in the early phase of *P. aeruginosa* biofilm development.

Initial studies to determine the capability of an *rhlA* mutant to migrate in conventional motility plate assays suggested that biosurfactants promote twitching motility of *P. aeruginosa*. The *P. aeruginosa rhlA* mutant used in the present study and other *P. aeruginosa rhlA* mutants of different PAO1 sublines were found to be impaired in twitching motility compared to their isogenic wild type (Fig. 1A and data not shown). The defect of the *rhlA* mutant strains in migration in the twitch plate assay could in each case be complemented by either introducing the biosurfactant biosynthesis genes *rhlAB* in *trans* or by providing the surfactant Tween 20 (Fig. 1B and C and data not shown). The observed defect of *P. aeruginosa rhlA* mutants in migration in twitch plate assays was to some extent surprising, since so far biosurfactants have only been reported to facilitate motility by *P. aeruginosa* in swarm plate assays (14, 29). However, it appears that the ability of biosurfactants to reduce surface tension and act as wetting agents may facilitate different kinds of surface-associated translocation of *P. aeruginosa*. In addition, it has been reported that biosurfactants may affect the composition of lipopolysaccharide (LPS) on the outer membrane of *P. aeruginosa* (2) and that a change in the LPS composition of *P. aeruginosa* may cause a significant reduction in the capability of the bacterium to migrate in both twitch plate and swarm plate assays (1). An effect of changes in cell surface LPS composition on migration in motility plate assays has been observed for several species, including *Salmonella enterica*, *Proteus mirabilis*, and *Myxococcus xanthus* (6, 17, 35, 53, 55). In the case of *S. enterica*, it was shown that the defect in motility due to changes in the LPS composition could be restored by providing a surfactant (53).

To investigate the impact of biosurfactants on migration-mediated structural biofilm development, we performed experiments with mixed-strain color-coded biofilms. Our results provide evidence that biosurfactants produced by *P. aeruginosa* indeed facilitate bacterial migration and colonization of the upper part of the biofilm. The *rhlA* mutant developed mushroom caps of reduced size on top of mushroom stalks formed by the *pilA* mutant, compared to the mushroom caps developed by the wild type in an analogous setup. In addition, experiments with three-strain color-coded biofilms suggested that, unlike the wild-type strain, the motility-impaired *rhlA* mutant was not able to colonize the top of the mushroom-shaped structures. These experiments also indicated that the possible effects of biosurfactant on cellular migration and structure formation are largely confined to the subpopulations that produce the biosurfactants. It is likely that, due to their inherent amphipathic characteristics, the biosurfactants will adhere directly to the cell surfaces of the producing cells or to a cell surface in close proximity and exert their effect on cellular migration, leading to the spatial distribution of distinct subpopulations within the multicellular structures. Because biosurfactant production evidently may facilitate both swarming and twitching motility, the involvement of biosurfactants in facilitating migration-dependent structural development in *P. aeruginosa* biofilms does not provide information regarding the type of surface-associated motility occurring.

In agreement with our observations, Davey et al. (10) reported that a *P. aeruginosa rhlA* mutant formed a flat biofilm. Contrary to our suggestion, however, Davey et al. (10) suggested that the biosurfactants are not required for the formation of initial microcolonies but that they participate in the maintenance of the channels between the microcolonies once they are formed. When we used the same *P. aeruginosa rhlA* mutant as was used by Davey and colleagues we got essentially the same results as reported by them. That is, this *P. aeruginosa rhlA* strain indeed formed microcolonies and the channels between the microcolonies were subsequently colonized (data not shown). However, when we moved the *rhlA* mutation via phage transduction to a number of different PAO1 sublines, the newly constructed *rhlA* mutants were all deficient in microcolony formation as reported here. This suggests that the *rhlA* mutant used by Davey et al. (10) carries additional mutations that enable it to form microcolonies in the absence of biosurfactants. However, differences in media and experimental setup may also be the cause of these seemingly divergent results.

A role of *P. aeruginosa* biosurfactants in initial microcolony formation appears to be contrary to the established roles of

biosurfactants in maintaining channels between microcolonies (10) and in dispersal of biofilm (5, 24, 47). However, in support of the proposed role of *P. aeruginosa* biosurfactant in initial microcolony formation, it has been shown that low concentrations of rhamnolipid enhance the cell surface hydrophobicity of *P. aeruginosa* by causing a release of LPS from the cell surface (2, 56). An increase in cell surface hydrophobicity could increase the adhesiveness of the bacteria to a level which is critical for initial microcolony formation in biofilms. In support of this suggestion, Herman et al. (22) showed that addition of low concentrations of rhamnolipid induced the formation of multicellular aggregates in *P. aeruginosa* suspensions. It is possible that the bacteria in wild-type *P. aeruginosa* biofilms produce low amounts of biosurfactants, sufficient to facilitate microcolony formation, in the early phase of biofilm development when the cell concentration is still not high enough to constitute a quorum. When the cell concentration reaches higher levels, the production of high concentrations of *P. aeruginosa* biosurfactants is expected to be induced via quorum sensing, and these high concentrations of *P. aeruginosa* biosurfactants might have the proposed effects on cellular migration, channel maintenance, and biofilm dispersal. At present we cannot explain why the effect of biosurfactants on structure formation in developed biofilms seemed to be confined largely to biosurfactant-producing subpopulations, whereas biosurfactant production by the wild type in young wild-type–*rhlA* mixed biofilms apparently could facilitate microcolony formation of both the wild type and the *rhlA* mutant. However, this might be related to differences in the amount and composition of biosurfactants required in the different processes. Caiazza and coworkers recently described that the various biosurfactant compounds produced by *P. aeruginosa* have different effects on cell surface hydrophobicity and cellular migration (7).

Due to the occurrence of nutrient and oxygen gradients in biofilms, the top of the mushroom-shaped structures is believed to be a favored location, because the cells present there will receive more substrate from the bulk liquid than the cells situated within the multicellular structures or close to the substratum (12, 16, 30, 43, 54). It appears that only cells which are fully capable of type IV pilus-driven migration succeed in reaching the favorable top of the mushroom-shaped structures. Experiments to investigate a possible role of chemotaxis in coordinating the cellular migration involved in cap formation are under way in our laboratory.

In conclusion, the present study together with the studies of others suggest that *P. aeruginosa* biosurfactants have multiple roles in *P. aeruginosa* biofilm development: (i) they are necessary for initial microcolony formation, (ii) they facilitate surface-associated bacterial migration and thereby the formation of mushroom-shaped structures, (iii) they prevent colonization of the channels between the mushroom-shaped structures (10), and (iv) they play a role in biofilm dispersion (5, 47).

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Paper 2

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The Biofilm Matrix: A Sticky Framework

4

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Abstract

The extracellular matrix of structured microbial communities constitutes the framework that holds the component cells together. Although the presence of cell-to-cell interconnecting matrices appears to be a common feature of structured microbial communities, there is a remarkable diversity in the composition of these matrices. Compounds such as polysaccharides, fimbriae, mating pili, and extracellular DNA can all function as extracellular matrix components. In the present chapter we provide examples of the diversity of biofilm matrices.

Introduction

The formation and maintenance of structured microbial communities critically depends on the presence of extracellular substances that constitute cell-to-cell interconnecting matrices. Proliferation of sessile bacteria and the production of cell-to-cell interconnecting extracellular compounds lead to the formation of microbial biofilms. The extracellular matrix that surrounds the bacteria in biofilms is believed to offer protection against various adverse factors including protozoan predation in environmental settings (Matz and Kjelleberg, 2005) and host immune responses in medical settings (Costerton *et al.*, 1999). The production of the cell-to-cell interconnecting components in biofilms apparently is a cost each bacterium pays in order to contribute to the social activity of creating a protective biofilm domicile. Although the presence of a cell-to-cell interconnecting matrix appears to be a common feature of microbial biofilms, there is a remarkable diversity in the composition of these matrices. Many kinds of exopolymers, e.g. polysaccharide, protein, and DNA may function as biofilm matrix material. In addition to these exopolymers, outer membrane proteins and a variety of cell appendages such as fimbriae, pili, and flagella may also function as part of the biofilm matrix. The components of the biofilm matrix are usually, but not always, produced by the bacteria themselves.

In the present chapter we present examples of polysaccharides, proteins, and extracellular DNA as matrix components in biofilms. It appears that the expression of polysaccharides and proteins of the extracellular matrix in many cases is regulated through proteins which contain GGDEF and/or EAL domains. These proteins control intracellular levels of the cyclic dinucleotide c-di-GMP through diguanylate cyclase and phosphodiesterase

activity. c-di-GMP in turn acts as a second messenger and affects matrix production and the adhesiveness of the bacteria. For a thorough description of the regulatory mechanism involving GGDEF and EAL domain proteins the reader is referred to Dow *et al.*, this volume. The generation of extracellular DNA is evidently in many cases regulated by means of quorum-sensing which is a mechanism that enable bacteria to monitor their cell population density through the extracellular accumulation of signaling molecules. For thorough descriptions of quorum-sensing in Gram-negative and Gram-positive bacteria the reader is referred to Atkinson *et al.*, and Yarwood, this volume.

Polysaccharides as matrix components in biofilms

Extracellular polysaccharides are usually very important parts of biofilm matrices. The chemical composition and physical properties of the polysaccharides in biofilm matrices can vary greatly due to the type of monomer units, the kind of glycosidic linkages (e.g. β-1,4, β-1,3 or α-1,6), and the occurrence of different organic and inorganic substitutions. Here we address five types of polysaccharides that have been shown to play important roles in biofilm formation by a number of different bacterial species: cellulose, PNAG/PIA, PEL, PSL and VPS.

Cellulose

Cellulose is the most abundant polysaccharide in nature and is produced by both plants and bacteria. Bacterial cellulose biogenesis and the role of cellulose in biofilm formation has been described for a number of bacterial species including *Gluconacteobacter xylinus* (formerly called *Acetobacter xylinum*) *Sarcina ventriculi, Agrobacterium tumefaciens, Rhizobium leguminosarum, Escherichia coli, Salmonella* spp., and *Pseudomonas fluorescence* (Ausmees *et al.*, 1999; Deinema and Zevenhuizen, 1971; Matthysse *et al.*, 1995; Napoli *et al.*, 1975; Ross *et. al.*, 1991; Zogaj *et al.*, 2001; Spiers *et al.*, 2003). However, comparative sequence analyses indicate that many other bacteria including *Vibrio, Yersinia,* and *Burkholderia* species have the capacity to synthesize cellulose. In the present chapter we will focus on G. *xylinus, Salmonella* spp., and *P. fluorescence* as model organisms.

When G. *xylinus* is grown *in vitro* in a static broth culture it forms a thick cellulose-containing biofilm at the air/liquid interface (Schramm and Hestrin, 1954). In nature G. *xylinus* has predominantly been found on decaying fruits, where a cellulose-matrix surrounding the bacteria may protect against competitors and lethal effects of UV light. The native cellulose is synthesized as a long polymeric chain composed of β-1,4-linked D-Glucose units by a multimeric enzyme complex, which is located in the cytoplasmic membrane of G. *xylinus*. A single row of pore-like structures on the outer membrane along the longitudinal axis of the rod-shaped bacterium, called linear terminal-complexes, has been identified to be the site of extrusion of the native cellulose chain (Brown *et al.*, 1976; Kimura *et al.*, 2001). Several of these single glucan chains coalesce via van der Waals forces and hydrogen bonds to form crystalline microfibrils. An entangled mesh of these microfibrils produces a gelatinous structure which constitutes the major component of the biofilm matrix.

In G. *xylinus* and other cellulose-synthesizing bacteria, the genetic elements coding for cellulose synthesis are located in an operon consisting of four genes which are generally designated *bcsA, bcsB, bcsC* and *bcsD* (bacterial cellulose synthesis). The polymerization of

single monomer units of UDP-glucose to cellulose polymer is catalyzed by the cellulose synthase BcsA. Several transmembrane domains anchor the cellulose synthase BcsA in the cytoplasmic membrane. In spatial proximity with the BcsA enzyme the BcsB protein can be found. This protein regulates the activity of cellulose synthesis via binding of the second messenger c-di-GMP (Ross *et al.*, 1987). It has been proposed that a controlled release of c-di-GMP from BcsB to the BcsA enzyme may activate the cellulase synthase allosterically. Recent bioinformatic investigations suggest the localization of a binding site of c-di-GMP, called the PilZ domain, at the C-terminal end of the BcsA cellulose synthase (Amikam and Galperin, 2006). The functions of BcsC and BcsD have not been described yet. However, sequence analysis suggests that BcsC might be involved in pore formation through the cell wall, whereas BcsD might have an effect on cellulose crystallization (Saxena *et al.*, 1994; Wong *et al.*, 1990).

Synthesis of cellulose by enterobacteria such as *Salmonella* spp. and *E. coli* has been associated with the ability to form biofilms on abiotic surfaces, such as glass and polystyrene (Garcia *et al.*, 2004; Römling *et al.*, 2000; Zogaj *et al.*, 2001). High-level production of cellulose and proteinaceous curli fimbria by these organisms result in the formation of wrinkly or rough colonies on agar plates. If the bacteria are grown on rich agar medium (of low osmolarity), containing the diazo dye Congo Red, which binds to cellulose and curli fimbriae, they will form characteristic colonies which appear red, dry and rough (Hammar *et al*,. 1995; Römling *et al.*, 2000; Zogaj *et al.*, 2001). Regulation of the biosynthesis of cellulose and curli fimbriae evidently occurs through a complex coordinated pathway. The CsgD protein, a member of the LuxR superfamily of transcriptional regulators, is believed to regulate the synthesis of curli fimbria directly via transcriptional activation of the curli biosynthesis operon *csgDEFG-csgBAC* (Römling *et al.*, 2000; Gerstel *et al.*, 2003). In addition, CsgD stimulates the synthesis of cellulose indirectly via transcriptional activation of AdrA (Römling *et al.*, 2000; Zogaj *et al.*, 2001). AdrA is a two-domain protein consisting of an N-terminal MASE2 domain (Nikolskaya *et al.*, 2003) and a C-terminal GGDEF domain, capable of producing c-di-GMP (Simm *et al.*, 2004). High concentrations of c-di-GMP have been shown to induce the cellulose biosynthesis operon (*yhjRQbcsABZC-bcsEFG*) and thereby the excretion of cellulose from *Salmonella enterica* (Zogaj *et al.*, 2001; Solano *et al.*, 2002). Recent data indicate that in addition to AdrA, other GGDEF-domain containing proteins of *Salmonella* spp., are involved in modulating the level of the c-di-GMP, and thus biofilm formation (Garcia *et al.*, 2004; Kader *et al.*, 2006). Cellulose and curli fimbriae together form a highly inert matrix, which account for the strength and integrity of biofilms formed under various conditions by these enterobacteria (Garcia *et al.*, 2004; Römling *et al.*, 2000; Zogaj *et al.*, 2001).

When *P. fluorescens* was grown for extended periods in static liquid cultures, which contained numerous niches, spontaneous variants that colonized the different niches arose at high frequencies (Rainey and Travisano, 1998). One group of these variants, which colonized the air/liquid interface of the static cultures by forming a robust biofilm-pellicle, was termed "the wrinkly spreader" because the bacteria formed distinct wrinkled colonies on agar plates (Rainey and Travisano, 1998). This highly aggregative phenotype of the wrinkly spreader has been linked to the production of an acetylated form of cellulose and a proteinaceous fimbrial like attachment factor (Spiers *et al.*, 2003; Spiers and Rainey 2005).

Complementation experiments suggested that the cellulose fibers could interact with the lipopolysaccharide (LPS) of neighboring cells and that this interaction in conjunction with the proteinaceous attachment factor was responsible for the strength and integrity of the pellicle (Spiers and Rainey 2005). Studies of *P. fluorescens* in the spatially heterogeneous environment of a static culture have also shown that cheater variants, which benefit from the production of matrix compounds by neighboring bacteria without contributing themselves, arise at high frequencies in the biofilm pellicle (Rainey and Rainey, 2003). The frequency of cheaters in a biofilm in a given system is limited to the point where their presence damages the integrity of the biofilm too much. In general, the emergence of cheaters is the key problem for the evolution of microbial cooperation in biofilms. However, the predominantly clonal structure of the microcolonies in biofilms, combined with the re-establishment of them by single cells, acts as a purification mechanism to get rid of cheaters, and promotes the evolution of cooperation in biofilms (Kreft, 2004).

Production of the acetylated cellulose polymer in *P. fluorescens* was shown to be encoded by genes in the 10-gene *wss* operon, and on the basis of homology with the *G. xylinus bcs* genes the WssB, WssC and WssE proteins were identified as the bacterial cellulose synthase subunits (Spiers *et al.*, 2002; Spiers *et al.*, 2003). The WssG, WssH and WssI proteins are unique to the *P. fluorescens* cellulose operon, but their homology to the AlgF, AlgI and AlgJ proteins of *P. aeruginosa* (Franklin and Ohman 1996) suggests a role in the acetylation of the polymer. The production of the cellulosic polymer has been demonstrated to enhance the fitness of *P. fluorescens* during colonization of the rhizosphere (Gal *et al.*, 2003). The wrinkly spreader phenotype was found to be regulated by the GGDEF domain protein WspR, which consists of a C-terminal CheY-like receiver domain and an N-terminal GGDEF output domain. Mutagenesis showed that the activity of WspR was dependent on phosphorylation of the protein via gene products encoded by the *wsp* operon, and that the GGDEF domain was essential for the regulation of cellulose production. (Spiers *et al.*, 2003; Goymer *et al.*, 2006).

PNAG/PIA

The role of poly-N-acetylglucosamine as matrix component in biofilms has been extensively studied in two closely related Gram-positive bacteria; the coagulase-negative organism *Staphylococcus epidermidis* which synthesizes polysaccharide intercellular adhesin (PIA), and the coagulase-positive organism *Staphylococcus aureus* which synthesizes poly-N-acetylglucosamine (PNAG). More recently it has been found that PNAG/PIA-like polysaccharides are also synthesized by Gram-negative bacteria such as *E. coli, Yersinia pestis* and *Actinobacillus* sp. In addition, comparative genome sequence analysis has revealed that homologues of the poly-N-acetylglucosamine biosynthesis genes are present in many bacterial species including *Pseudomonas fluorescence, Bordetella pertussis, Ralstonia solanacearum,* and *Lactococcus lactis*. Here we will turn our attention on PNAG/PIA synthesized by *S. epidermidis, S. aureus, E. coli* and *Y. pestis*.

S. aureus and *S. epidermidis* are frequently found as harmless inhabitants of the mucosal nasal passages or the normal skin flora of humans. However, these organisms are opportunistic pathogens and they are increasingly found to be the cause of invasive, and chronic, medical device-associated infections. These implant-associated infections are difficult to

eradicate and it is believed that the biofilm mode of growth is responsible for the inherent tolerance towards host immune responses and antimicrobial treatment. Biofilm development by staphylococci is multifactorial. Many extracellular or surface-bound polymers have been identified to play a role in cell-to-cell adhesion and binding to biotic and abiotic surfaces, but in general PNAG/PIA is believed to be the most important biofilm matrix component for the staphylococci (Caiazza and O'Toole, 2003; Foster and Höök, 1998; Gross *et al.*, 2001; Götz, 2002; Vuong *et al.*, 2000).

The polysaccharides PIA and PNAG synthesized by *S. epidermidis* and *S. aureus*, are chemically and immunologically closely related. PIA and PNAG are both homopolysaccharides, composed of unbranched long polymeric chains of D-glucosamine uniformly linked together by α-1,6 glucosidic bonds, but they differ in chain length and modification (Mack *et al.*, 1996; Maira-Litran *et al.*, 2002). The wheat germ agglutinin lectin (WGA) was found to bind to N-acetylglucosamine produced by staphylococci (Jäger *et al.*, 2005; Sanford *et al.*, 1995; Sanford *et al.*, 1996; Thomas *et al.*, 1997). Figure 4.1 visualizes the

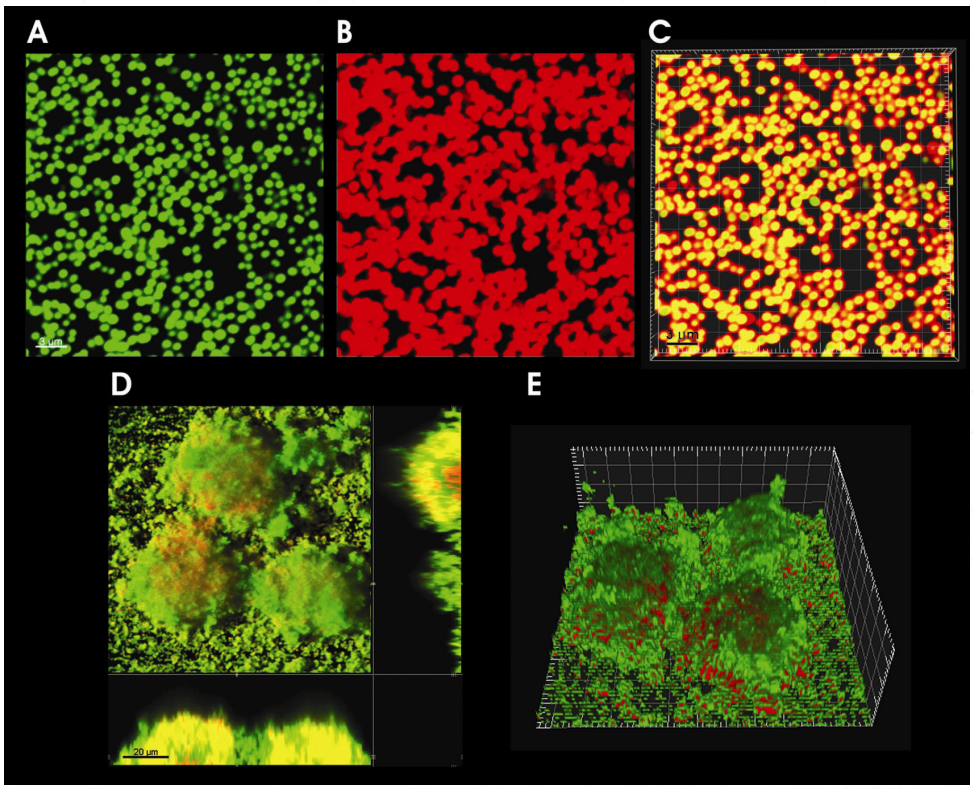

Figure 4.1 Confocal laser scanning micrographs showing bacterial cells (stained with Syto 9) and PNAG exopolysaccharide (stained with WGA-TMR) in a biofilm formed by *Staphylococcus aureus* SJ235 (a clinical isolate obtained from a cystic fibrosis patient). Staphylococcal cells were cultivated in a continuous flow chamber setup perfused with minimal medium. After 24 hours of cultivation, the cells had formed a single-layer biofilm on the glass surface (A), and PNAG could be visualized (B). An overlay of the two images demonstrates that most cells were surrounded by PNAG exopolysaccharide (C). After 3 days of cultivation, a biofilm with microcolonies containing staphylococci and PNAG had formed as visualized by the top-down shadow projection with two flanking section images (D) and with the 3D image projection (E).

presence of the extracellular polysaccharide in a staphylococcal biofilm using fluorescently labeled WGA lectin and confocal laser scanning microscopy.

The genetic elements coding for the biosynthesis of poly-N-acetylglucosamine are located in an operon consisting of the four genes *icaA*, *icaD*, *icaB* and *icaC* (intercellular adhesin). A gene, *icaR*, located upstream of *icaADBC* and transcribed divergently, encodes a transcriptional repressor of the *icaADBC*-operon (Conlon *et al.*, 2002a; Jefferson *et al.*, 2004; Cramton *et al.*, 1999; Heilmann *et al.*, 1996; Gerke *et al.*, 1998). IcaA, IcaC and IcaD are located in the cellular membrane, whereas IcaB can be found extracellularly. The polymerization of single monomer units of UDP-N-acetylglucosamine to polymer chains of β-1,6-N-acetylglucosamine is catalyzed by the N-acetylglucosaminyl-transferase IcaA. Co-expression of *icaA* with *icaD* increased the enzyme activity significantly, indicating a supportive function of IcaD for IcaA enzyme activity. Oligomers of N-acetylglucosamine produced by IcaAD only reach a maximal length of 20 residues. Expression of long N-acetylglucosaminyl chains requires co-expression of *icaA* and *icaD* with *icaC* (Gerke *et al.*, 1998). TcaR (Teicoplanin associated regulator), a protein with sequence homology to MarR-like transcriptional regulators, was shown to influence transcription of *icaADBC* negatively in *S. aureus* (Jefferson *et al.*, 2004). SarA (staphylococcal accessory regulator A), a global regulator for expression of virulence factors in *S. aureus*, was shown to upregulate transcription of *icaADBC* in *S. aureus* as well as *S. epidermidis* (Beenken *et al.*, 2003; Tormoet *al.*, 2005b; Valle *et al.*, 2003). Staphylococcal cells lacking SarA showed reduced binding to abiotic surfaces such as polystyrene or glass in static as well as continuous flow systems (Beenken *et al.*, 2003; Tormo *et al.*, 2005b; Valle *et al.*, 2003). Quorum sensing in staphylococci (encoded by the accessory gene regulator (*agr*) locus) was shown to influence biofilm formation of both *S. aureus* and *S. epidermidis*, but apparently through an *ica*-independent pathway (Vuong *et al.*, 2000; Vuong *et al.*, 2003; Yarwood *et al.*, 2004).

The alternative sigma factor B (σ^B) is part of a complex regulatory network that regulates basic cellular processes as well as virulence factor expression in *S. aureus* and possibly also in *S. epidermidis* (Kies *et al.*, 2001; Novick, 2003; Pane-Farre *et al.*, 2006; Ziebandt *et al.*, 2001). Previously σ^B was found to regulate biofilm formation of an *S. aureus* mucosal isolate (Rachid *et al.*, 2000a), but more recent data indicate that σ^B might play a minor role in regulating biofilm development of *S. aureus* as complete deletion of *sigB* did not significantly affect PNAG production (Valle *et al.*, 2003). In contrast, a positive, although indirect, influence of σ^B on PIA regulation seems to be established for *S. epidermis*. Deletion of either σ^B itself or its positive regulator *rsbU* was shown to decrease production of PIA and therefore lead to a biofilm-negative phenotype in a microtiter tray assay (Knobloch *et al.*, 2001; Knobloch *et al.*, 2004). The biofilm-negative phenotype could be restored by addition of ethanol resulting in downregulation of *icaR* and increased synthesis of PIA (Knobloch *et al.*, 2001; Knobloch *et al.*, 2004), indicating the presence of an additional regulatory element in the pathway that regulates PIA synthesis.

It has been widely observed that biofilm formation of staphylococci is enhanced in the presence of additional NaCl, glucose, and ethanol. While the glucose- and NaCl-dependent biofilm formation in *S. aureus* seems to be regulated independently of the *ica*-locus, enhanced biofilm formation of *S. epidermidis* in the presence of NaCl or ethanol is regulated via transcriptional induction of *icaADBC* (Conlon *et al.*, 2002a; Conlon *et al.*, 2002b; Lim

et al., 2004). Addition of glucose seems to affect the biosynthesis of PIA in *S. epidermidis* positively, but at a stage subsequent to *icaADBC* transcription (Mack *et al.*, 1992; Dobinsky *et al.*, 2003). Of therapeutic relevance is the observation that some antibiotics are able to induce PIA production in *S. epidermidis*. Subinhibitory concentrations of tetracycline and quinuprisin-dalfopristin were found to induce transcription of the *ica*-operon and promote biofilm formation in a microtiter tray assay (Rachid *et al.*, 2000b).

E. coli is, in addition to cellulose, colanic acid and capsular polysaccharides, able to synthesize the PNAG/PIA-like polysaccharide PGA (poly-N-acetyl-glucosamine). Disruption of the *pga* locus in *E. coli* was found to decrease biofilm formation significantly in a polystyrene microtiter tray assay (Blattner *et al.*, 1997; Wang *et al.*, 2004). The PGA exopolymer produced by *E. coli* consists of unbranched poly-β-1,6-N-acetylglucosamine, contains less than 3% non-N-acetylated glucosaminyl moieties and does not possess any major substitutions (Wang *et al.*, 2004). The genetic elements coding for the biosynthesis of PGA are located in an operon consisting of the four genes *pgaABCD* (formerly called *ycdSRQP*) (Blattner *et al.*, 1997; Wang *et al.*, 2004). The low G+C content of the *pgABCD* locus in *E. coli* (44% versus 51%) suggests that these genes were horizontally transferred (Wang *et al.*, 2004). PgaC is an N-glycosyltransferase which is predicted to be anchored in the membrane by two N-terminal and three C-terminal transmembrane domains. PgaB is assumed to be a lipoprotein and shares sequence similarities with the staphylococcal protein IcaB (Wang *et al.*, 2004). The protein sequence of PgaA indicates that it is located in the outer membrane, suggesting that it might participate in mediating translocation of PGA to the cell surface. PgaD is supposed to be a small inner membrane protein with two N-terminal membrane-spanning domains (Wang *et al.*, 2004).

The *pgaABCD* operon in *E. coli* was shown to be under negative control of CsrA (global storage regulator A). Disruption of *csrA* in *E. coli* led to a significant increase in biofilm formation in microtiter trays and on a glass surfaces (Jackson *et al.*, 2002). CsrA is a small RNA-binding protein which is a central effector molecule in the global regulatory system Csr, which controls bacterial gene transcription on the post-transcriptional level (Romeo, 1998). CsrA is known to be involved in regulating central metabolic pathways, such as glycogen synthesis and catabolism, gluconeogenesis, and glycolysis (Sabnis *et al.*, 1995; Yang *et al.*, 1996). CsrA binds to the *pgaA* mRNA in competition with the ribosome and prevents its translation by destabilizing the transcript (Romeo, 1998). Six putative CsrA binding sites in the *pgaA* mRNA leader have been identified (Wang *et al.*, 2005). CsrB and CsrC, two small RNAs (sRNAs), have been found to antagonize the regulatory effects of CsrA. These sRNAs have repeat sequences and were shown to bind several CsrA proteins, thereby sequestering CsrA from its target mRNA leader. This mechanism leads to positive modulation of the transcription of *pgaABCD* and therefore PGA synthesis (Weilbacher *et al.*, 2003). Recent research indicates that sRNAs in general may be key regulators of virulence factors and adaptive processes in Gram-negative as well as Gram-positive pathogens (Romby *et al.*, 2006).

The *hmsHFRS* (hemin storage) genes of *Y. pestis* share similarities to the *icaADBC* genes of staphylococci (Darby *et al.*, 2002; Pendrak and Perry, 1993; Perry *et al.*, 1990), and were shown to be required for biofilm formation by *Y. pestis* in a flow-chamber system (Jarrett *et al.*, 2004). The *hmsHFRS* operon is located on a 102 kb large mobile high-patho-

genicity island (HPI) which has been found in *Y. pestis* and *Y. pseudotuberculosis* (Buchrieser *et al.*, 1999; Fetherston *et al.*, 1992). Sequence analysis predicts that HmsH is located in the outer membrane of *Y. pestis*, whereas HmsF is supposed to be a lipoprotein (Lillard *et al.*, 1997). HmsR has about 39% identity to the staphylococcal N-glycosyltransferase IcaA, and HmsS is predicted to be a small inner membrane protein with two N-terminal membrane-spanning domains (Darby *et al.*, 2002; Lillard *et al.*, 1997). Evidence has been presented that expression of the *hsmHFRS* locus is regulated in a temperature dependent fashion via the HmsT and HmsP proteins, which contain a GGDEF and an EAL domain, respectively (Kirillina *et al.*, 2004).

Biofilm formation allows *Y. pestis* to colonize the proventricular valve within fleas (Darby *et al.*, 2002; Hinnebusch *et al.*, 1996; Jarrett *et al.*, 2004). Colonization of the proventricular valve, which separates the midgut from the esophagus, causes physical blockage of the flea, and efficient transmission of *Y. pestis*, the etiological agent of plague, to humans and rodents is facilitated when a blocked flea attempts to feed (Darby *et al.*, 2002; Hinnebusch *et al.*, 1996; Jarrett *et al.*, 2004).

PEL and PSL

P. aeruginosa can, dependent on the strain and growth conditions, produce at least three different polysaccharides: alginate, PEL and PSL. Besides these, two additional gene clusters which putatively are involved in polysaccharide biosynthesis (PA1381-PA1398 and PA3552-PA3558) have been identified in the chromosome of the reference strain *P. aeruginosa* PAO1. Mucoid forms of *P. aeruginosa* that over-express the *alg* genes (alginate biosynthesis, PA3540-PA3551) are primarily found in infected lungs of cystic fibrosis patients (Govan and Deretic, 1996). However, most *P. aeruginosa* strains are non-mucoid, and alginate was found not to be a significant component of the extracellular matrix of non-mucoid laboratory strains (Wozniak *et al.*, 2003). In the present chapter we will describe PEL and PSL as examples of exopolysaccharides in the extracellular matrix of *P. aeruginosa* biofilms.

While PEL and PSL both seem to be branched heteropolysaccharides, the main component of PEL is glucose, whereas PSL has a high content of mannose (Friedman and Kolter, 2004a; Friedman and Kolter, 2004b). High-level expression of PEL and PSL in *P. aeruginosa* was shown to lead to the formation of wrinkly colonies on agar plates, and synthesis of PEL was shown to enable *P. aeruginosa* to form pellicle-biofilm at the air/liquid interface of broth cultures (Friedman and Kolter, 2004a; Friedman and Kolter, 2004b). Proteinaceous cup fimbriae appear to participate together with PEL in *P. aeruginosa* biofilm formation under some conditions (Friedman and Kolter, 2004a), similar to the cellulose and curli fimbriae-containing matrix of *Salmonella* sp. biofilms described above. Studies using a static attachment assay provided evidence that PSL is important in the early stages of *P. aeruginosa* biofilm development, whereas the synthesis of PEL seems to be important in later stages of biofilm development (Friedman and Kolter, 2004b; Vasseur *et al.*, 2005). In a continuous culture flow-chamber set-up, a *P. aeruginosa* strain deficient in PSL production was found to be impaired in biofilm formation (Jackson *et al.*, 2004; Matsukawa and Greenberg, 2004), supporting a role of PSL in the early stages of biofilm development.

The genetic elements encoding the biosynthesis of PEL are organized in a gene cluster which consists of seven open reading frames termed *pelA-G* (pellicle formation; PA3058-PA3064), spanning a 12.2 kb region of the *P. aeruginosa* genome. Although the exact function of the gene products is not described yet, sequence analysis revealed that these proteins contain domains, which are present in proteins involved in polysaccharide processing in other organisms. For example, PelF shares sequence homology with group IV glycosyl transferases, PelA has similarity to endo α-1,4 polygalactosaminidase, and PelE contains domains which resembles those of sucrose synthases. In addition, PelG was suggested to be a PST-family protein, whose members are involved in translocating glycolipid precursors through the membrane. Many of the proteins contain transmembrane domains, indicating their final location in the membrane (Friedman and Kolter, 2004a; Vasseur *et al.*, 2005).

The *psl* (polysaccharide synthesis locus) gene cluster contains the 15 co-transcribed open reading frames *pslA-O* (PA2231-PA2245). Sequence analysis of the first 11 predicted gene products revealed homologies to proteins involved in polysaccharide biosynthesis. The putative gene products PslF, PslH and PslI share sequence homologies with group I family glycosyltransferases, whereas PslA is similar to sugar transferases and PslD might be involved in polysaccharide transport. Since PslA, PslJ and PslK are predicted to contain 6, 11 and 12 transmembrane domains respectively, they are most likely located in the cellular membrane (Friedman and Kolter, 2004b; Jackson *et al.*, 2004; Matsukawa and Greenberg, 2004)

Recent data indicate that synthesis of the polysaccharide matrix in *P. aeruginosa* biofilms is regulated via intracellular levels of c-di-GMP. Transcription of the *pel* and *psl* loci were found to be regulated through the *wsp* chemosensory system (*wspABCDEFR*, PA3708-PA3702), of which the two gene products, WspR and WspF contain the catalytic GGDEF and EAL domains respectively. Whereas high levels of c-di-GMP were found to stimulate transcription of the *pel* and *psl* loci and induce biofilm formation, low intracellular c-di-GMP levels were found to decrease biofilm formation in a microtiter tray assay and in a flow-chamber system (Hickman *et al.*, 2005).

VPS

Vibrio cholerae produces the exopolysaccharide VPS (Vibrio polysaccharide) which causes the formation of wrinkly (rugose) colonies on agar plates and has been shown to have an important role in biofilm formation on solid surfaces and at liquid–air interfaces (Casper-Lindley and Yildiz, 2004; Wai *et al.*, 1998; Watnick and Kolter, 1999; Yildiz *et al.*, 2001; Yildiz and Schoolnik, 1999). The bacteria in *V. cholerae* biofilms have been shown to exhibit an enhanced survival in chlorinated water, and an elevated tolerance towards osmotic, acid and oxidative stresses compared to their planktonic counterparts (Morris *et al.*, 1996; Wai *et al.*, 1998; Yildiz and Schoolnik, 1999). The polysaccharide produced by *V. cholerae* contains mainly glucose and galactose as monomeric sugar components, and in addition smaller amounts of N-acetylglucosamine and mannose (Yildiz and Schoolnik, 1999).

The genetic elements coding for the biosynthesis of VPS are organized in two gene clusters, *vspI* (*vpsA-K*, VC0917-VC0927) and *vspII* (*vpsL-Q*, VC09334-VC0939), that encompass a 30.7 kb region on the large chromosome of *V. cholera*. Sequence analysis revealed that the gene products of the *vps* gene cluster share sequence similarities with

proteins involved in exopolysaccharide synthesis and polysaccharide modification in other bacterial species, among these glycosyl transferase, UDP-glucose dehydrogenase, glycosyl-1-phosphate transferase and NDP-N-acetyl-D-galactosaminuronic acid dehydrogenase (Yildiz and Schoolnik, 1999). *In vitro* analysis demonstrated that transcription of the gene clusters *vpsI* and *vpsII* are regulated positively by the two response regulators VpsR and VpsT. Knock-out mutants in either *vpsR* or *vpsT* or both genes displayed reduced biofilm formation in a polyvinyl microtiter tray assay and in a static borosilicate glass slide assay compared to the rugose isogenic wild-type strain. VpsR belongs to the NtrC subclass of transcriptional regulators, whereas VspT shares sequence homologies with the transcriptional regulators CsgD and AgfD of *E. coli* and *Salmonella* sp. (Casper-Lindley and Yildiz, 2004; Yildiz *et al.*, 2001).

Evidence has been provided that transcription of the *vps* genes in *V. cholerae* is regulated through the second messenger c-di-GMP (Rashid *et al.*, 2003; Tischler and Camilli, 2004). Whole genome transcriptional studies of bacteria which formed smooth and rugose colonies, respectively, showed that five genes which code for proteins containing GGDEF and/or EAL domains were differentially expressed in the two colonial phenotypes (Yildiz *et al.*, 2004). More detailed analysis demonstrated that the GGDEF/EAL domain proteins CdgC (cyclic diguanylate), RocS (regulation of cell signaling) and MbaA (maintenance of biofilm architecture) all regulate *vps* transcription and *V. cholerae* biofilm formation negatively via the VpsR regulator (Bomchil *et al.*, 2003; Lim *et al.*, 2006; Rashid *et al.*, 2003). In addition, high intracellular levels of the EAL domain-containing protein AcgA was shown to decrease *V. cholerae* biofilm formation, whereas high intracellular levels of the GGDEF domain-containing protein AcgB was shown to increase *V. cholerae* biofilm formation in a microtiter tray assay. Whether regulation of *V. cholerae* biofilm formation via AcgA and AcgB is dependent on the *vps* genes remains to be investigated (Kovacikova *et al.*, 2005).

Several environmental stimuli and complex regulatory pathways are involved in the aquatic and intestinal life cycles of *V. cholera*. Along with the synthesis of the cholera toxin (CT) and toxin co-regulated pili (TCP), the synthesis of the extracellular VPS matrix seems to play a major role in the life cycle and pathogenesis of *V. cholera*. It appears that quorum sensing negatively regulates biofilm formation as well as CT and TCP expression in *V. cholera*. At low cell density, the transcriptional regulator LuxO (together with the alternative sigma factor σ^{54}) activates expression of four small regulatory RNAs. These sRNAs together with Hfq repress HapR (homologue to LuxR of *V. harveyi*) expression by destabilizing the *hapR* mRNA. In the absence of HapR the genes *vps*, *ctx* (cholera toxin) and *tcp* (toxin co-regulated pilus) are expressed. At high cell density, LuxO is inactive and therefore repression of *hapR* is relieved, and HapR negatively regulates biofilm formation as well as *ctx* and *tcp* expression (Hammer and Bassler, 2003; Jobling and Holmes, 1997; Lenz *et al.*, 2004; Vance *et al.*, 2003; Zhu and Mekalanos, 2003; Zhu *et al.*, 2002).

Recent investigations indicate that the biofilm growth mode of *V. cholera* might play an important role in the transmission of the diarrheal disease cholera (Zhu and Mekalanos, 2003; Faruque *et al.*, 2006). *V. cholera* cells have been found to exist as aggregates (biofilms) of partially dormant cells in surface waters (Faruque *et al.*, 2006). Within these aquatic environments the biofilm mode of growth might increase survival of *V. cholera*, for instance against grazing by protozoa as has been shown by Matz *et al.* (2005). Upon oral ingestion,

these biofilms might furthermore be protected against acid stress in the gastric area. In the intestinal environment single cells might detach from the biofilm, due to high levels of quorum-sensing signal. In the intestinal environment with low quorum-sensing levels, expression of CT and TCP is enhanced and colonization of the intestinal sites induced. Again, high cell densities lead to high levels of quorum-sensing signal and repression of VPS, CT and TCP but induction of proteases, which might facilitate detachment of cell aggregates and single cells from the intestinal sites and exit from the host (Zhu and Mekalanos, 2003). In agreement, single free-swimming cells and small aggregates have been observed in stools from cholera patients (Faruque *et al.*, 2006).

Proteins as matrix components in biofilms

Genetic and microscopic approaches have provided information about proteins that play roles as cell-to-cell interconnecting factors in the course of biofilm formation by different bacterial species. In the present chapter we present examples from two different groups: multimeric cell appendages and surface proteins.

Multimeric cell appendages

Large multimeric cellular appendages such as flagella, fimbriae, and pili typically consist of numerous major structural protein components and several auxiliary proteins.

In many bacterial species flagella play a role in the initial phase of biofilm formation under some conditions (e.g. O'Toole and Kolter 1998a, b; Klausen *et al.*, 2003b; Watnick and Kolter, 1999), and they may also play a role in the later phases of structural biofilm development (Klausen *et al.*, 2003b; Yamada *et al.*, 2005). It appears that flagellum-driven motility can promote initial biofilm formation by facilitating transport of the bacteria to a surface (Gilbert *et al.*, 1993). In addition, evidence has been provided that flagella can act as both cell-to-surface adhesins and cell-to-cell adhesins (O'Toole and Kolter 1998a; Yamada *et al.*, 2005).

Type IV pili are used by a number of bacteria to perform surface associated motility (Mattick, 2002). In addition type IV pili have been shown to mediate adhesion to both abiotic and biotic surfaces under some conditions (Giltner *et al.*, 2006; Mattick, 2002; Sheth *et al.*, 1994; Schweizer *et al.*, 1998; O'Toole and Kolter 1998a). *In vitro* studies revealed that type IV pili of *P. aeruginosa* display specificity towards asialo-GM1 and asialo-GM2 on host cell surfaces (Hahn, 1997; Gupta *et al.*, 1994; Ramphal *et al.*, 1991). Recently type IV pili of *Neisseria gonorrhoeae* and *P. aeruginosa* were shown to bind with high affinity to DNA (Aas *et al.*, 2002; van Schaik *et al.*, 2005), and because extracellular DNA has been shown to be part of the extracellular matrix material in *P. aeruginosa* biofilms (Whitchurch, *et al.*, 2002; Nemoto *et al.*, 2003; Matsukawa and Greenberg, 2004; Allesen-Holm *et al.*, 2006), type IV pili might act as crosslinkers between the cells and the extracellular DNA matrix. Sauer and Camper (2001) presented evidence that expression of the major structural component of type IV pili, PilA, is downregulated in the initial stages of biofilm formation, but upregulated in the later stages of biofilm development.

Type 1 fimbriae-like organelles are encoded by various members of the enterobacteria and they are believed to play an important role during pathogenesis of some of these organisms (Abraham *et al.*, 1988). Each bacterial cell typically carries 100–500 type 1 fimbriae

on its surface (Hahn *et al.*, 2002). Type 1 fimbriae consist primarily of the structural protein FimA, but several auxiliary proteins are necessary for transport and assembly of the structural proteins (Klemm 1984; Klemm and Christiansen 1990; Klemm 1992). A minor component of type 1 fimbriae is the mannose specific adhesin FimH which is responsible for the ability of type 1 fimbriae to bind to eukaryotic cells (Krogfelt *et al.*, 1990). Evidence has been presented that type 1 fimbriae play a role in the formation of *E. coli* biofilms at the air–liquid interface in static liquid cultures (Duguid *et al.*, 1966; Pratt and Kolter 1998). The formation of these pellicles was inhibited by addition of mannose derivatives, suggesting that the process was dependent on the adhesive functions of the FimH adhesin (Old and Duguid, 1970; Harris *et al.*, 1990). Type 1 fimbriae have also been associated with increased biofilm formation by *E. coli* in flow-chamber systems (Schembri and Klemm 2001). These observations suggest that type 1 fimbriae, besides mediating binding to eukaryotic cell surfaces, may also act as matrix components in biofilms.

Mutations in genes encoding *P. aeruginosa* cell appendages termed Cup fimbria were shown to affect the ability of the cells to attach to surfaces in microtiter trays (Vallet *et al.*, 2001). Evidence is accruing that Cup fimbriae in addition to their role in initial biofilm formation also play a role as cell-to-cell interconnecting compounds in mature biofilms. The *sadARS* genes code for a putative sensor histidine kinase and two response regulators, and mutations in any of these genes were shown to result in the formation of *P. aeruginosa* biofilms with an altered mature structure (Kuchma *et al.*, 2005). In another study the *sadARS* genes (termed *rocARS*) were shown to regulate transcription of the CupB and CupC fimbriae through the action of the positive regulator RocA, and the negative regulator RocR, which contain an EAL domain (Kulasėkara *et al.*, 2005). D'Argenio and colleagues demonstrated that expression of CupA fimbriae was necessary for the formation of wrinkly colonies by a *P. aeruginosa* mutant, and that the GGDEF domain protein WspR was required for this phenotype (D'Argenio *et al.*, 2002).

It appears that the presence of conjugative pili may promote biofilm formation in *E. coli*. Ghigo (2001) observed that strains harboring and expressing conjugative plasmids displayed an increase in biofilm formation on Pyrex slides placed in microfermentors. In a flow-chamber system the presence of conjugative pili on the surface of *E. coli* K-12 cells was shown to be the critical biofilm matrix component whereas other known *E. coli* biofilm formation factors like Ag43 and type 1 fimbria were dispensable (Reisner *et al.*, 2003). Even minor changes of the conjugative pili structure, such as those conferred by a deletion of the *traX* gene, resulted in either the formation of biofilms with altered spatial structure, or in a decrease in biofilm formation (Reisner *et al.*, 2003).

Curli were first identified in *E. coli* but subsequent studies have shown that they are also produced by *Salmonella*, *Citrobacter* and *Enterobacter* species (Prigent-Combart *et al.*, 2000; Zogaj *et al.*, 2003). Curli are thin amyloid-like structures protruding from the cell surface as a tangled amorphous matrix, and they may function as both cell-to-surface and cell-to-cell adhesins (see Figure 4.2) (Vidal *et al.*, 1998; Römling *et al.*, 1998; Prigent-Combart *et al.*, 2000). The CsgA protein is the primary structural component of curli and the CsgB protein is an important minor structural unit. Polymerization of curli occurs outside the cell in a process referred to as extracellular nucleation. This nucleation is dependant of the CsgB protein and mutant studies have shown that mixing of *csg*A and *csg*B mutants can

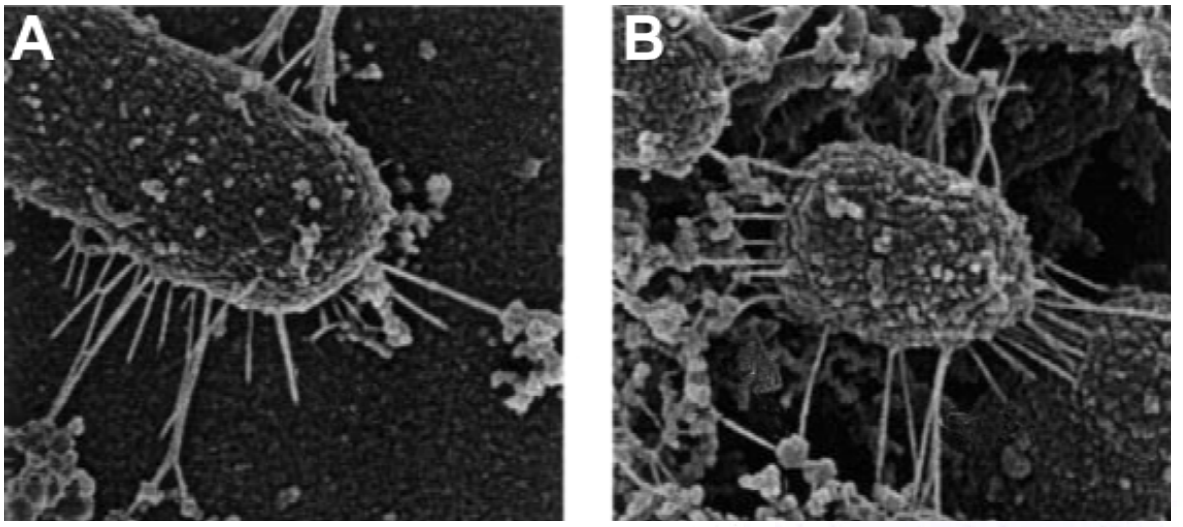

Figure 4.2 Scanning electron micrographs showing curli-mediated adherence of *E. coli* cells to a surface (A) and to each other (B). Adapted from Environ. Microbiol. 2:450–464 with permission from Blackwell Publishing.

result in the precipitation of curli subunits on the surface of the *csgA* mutant, suggesting that the CsgA protein is freely diffusible in the extracellular surroundings. In addition to binding to abiotic surfaces and stimulating biofilm formation by mediating cell-to-cell adherence, curli have been demonstrated to interact with host cell exopolymers such as fibronectin (Olsen *et al.*, 1989; Austin *et al.*, 1998). Evidence has been provided that curli together with cellulose play a role for biofilm formation by members of the enterobacteria such as *E. coli*, *Enterobacter* and *Citrobacter* (Zogaj *et al.*, 2003; Bokranz *et al.*, 2005). In *E. coli* and *S. enterica* it has been shown that the coordinated expression of curli and cellulose is regulated through the transcriptional regulator CsgD and the GGDEF domain protein AdrA as described above (Prigent-Combaret *et al.*, 2001; Römling *et al.*, 2000; Garcia *et al.*, 2004; Kader *et al.*, 2006).

Surface proteins

In *E. coli* a group of surface proteins (e.g. Ag43, AIDA and TibA) termed self associating autotransporters or SAAT has been identified (Diderichsen 1980; Benz and Schmidt 1992; Lindenthal and Elsinghorst, 1999; Klemm *et al.*, 2006). These proteins all have sequence similarities and share common features such as promotion of cell aggregation and biofilm formation (Henderson *et al.*, 1997; Danese *et al.*, 2000). Cell-to-cell interconnection mediated by these surface proteins occurs by virtue of there self-recognizing nature (Klemm *et al.*, 2004; Henderson *et al.*, 1997).

The TasA protein of *Bacillus subtilis* was recently recognized as a major component of the extracellular matrix surrounding *B. subtilis* cells during formation of biofilms at the liquid-air interface (Branda *et al.*, 2006). Based on genetic and biochemical evidence it was demonstrated that biofilm formation, in addition to the TasA protein, required an exopolysaccharide component encoded by the *epsA-O* operon, and that the absence of both of these components led to the abolishment of *B. subtilis* biofilm formation (Branda *et al.*, 2001; Branda *et al.*, 2006). Inactivation of either component alone resulted in a residual biofilm matrix and extracellular complementation was possible through mixing of the two mutant

strains. Evidence has been provided that the *eps* and *tasA* genes are coregulated through the *B. subtilis* regulator SinR (Chu *et al.*, 2006). The exact function of TasA in the extracellular matrix of *B. subtilis* remains unknown, but in addition to its role in biofilm formation it appears to have broad-spectrum antibacterial activity (Stover and Driks, 1999), and seems to be part of the spore coat assembly and germination (Serrano *et al.*, 1999).

Lectins are characterized by affinity towards carbohydrate residues on host cell surfaces, but evidence has been provided that some lectins recognize carbohydrates in extracellular biofilm matrices and thereby promote cell-to-cell interconnection. The *P. aeruginosa* fucose specific LecB lectin has recently been demonstrated to be important for the development of flow-chamber grown biofilms (Loris *et al.*, 2003; Tielker *et al.*, 2005). Cell fractionation experiments suggested that LecB was exported and bound to the outer-membrane through interaction with fucose containing ligands (Tielker *et al.*, 2005). Staining of *P. aeruginosa* biofilms with fluorescently labeled LecB protein confirmed the presence of lectin binding sites in these structures (Tielker *et al.*, 2005). The galactophilic lectin, LecA, has also recently been shown to have a role in biofilm development by *P. aeruginosa* (Diggle *et al.*, 2006).

The search for factors involved in biofilm formation has revealed the widespread existence of a large group of high molecular weight surface proteins that share limited sequence homology but are characterized by extensive repeat structures (Lasa and Penadés, 2005). Although the repetition of domains is central to the grouping of these diverse proteins, this might only reflect a general requirement for such surface proteins to protrude from the bacterial surface. Repetitive protein sequences could reflect a number of structural units necessary for obtaining protrusion from the surface and could otherwise be unrelated to the biological function of the individual proteins of the group. In addition to the repetition of domains, these proteins share a number of functional characteristics such as promotion of cell aggregation, surface adhesion and biofilm formation. The protein family includes the biofilm-associated protein (Bap) of *Staphylococcus aureus*, the large adhesion protein (LapA) of *P. fluorescens* and *P. putida*, the biofilm associated protein (BapA) of *Salmonella enterica*, the enterococcal surface protein (Esp) of *Enterococcus faecalis*, and the AdhA adhesin of *Burkholderia cenocepacia*. In addition to these experimentally characterized proteins a large number of similar proteins are found in the genome databases of both environmental and medically relevant bacteria.

The Bap protein was first described in a study with an *S. aureus* bovine mastitis isolate, and was found to be essential for biofilm formation by this organism (Cucarella *et al.*, 2001). Bap was shown to promote both primary attachment to abiotic surfaces and cell-to-cell adhesion. In addition, evidence was presented that deletion of the *bap* gene was linked to a decreased accumulation of the major staphylococcal exopolysaccharide, PIA. Bap appears to be almost universally conserved in biofilm forming *S. aureus* isolates, and has also been linked to the pathogenesis of bovine isolates (Cucarella *et al.*, 2004). Close homologs of Bap have been found in numerous other staphylococcal species among these *S. epidermidis* (Tormo *et al.*, 2005a).

Mutations in the *bapA* gene of *S. enterica* were shown to abolish biofilm formation, but the defect could be rescued by overexpression of curli (Latasa *et al.*, 2005). The *bapA* gene is located next to genes encoding a putative type 1 transport system and the BapA protein

might be exported to the surface in a process similar to that identified for the LapA protein of *P. fluorescens* (described below) (Lasa and Penadés, 2005). The expression of the BapA protein has been proposed to be co-regulated with the other two major components of the *S. enterica* matrix, curli and cellulose, through the CsgD regulatory protein (Latasa *et al.*, 2005).

In the case of the Esp protein of *E. faecalis* it was shown by in-frame deletions that only the non-repetitive N-terminal domain was required for enhancement of biofilm formation (Toledo-Arana *et al.*, 2001; Tendolkar *et al.*, 2004), and that a fusion between the N-terminal region and a heterologous anchoring protein was sufficient to restore biofilm formation (Tendolkar *et al.*, 2005). Expression of *esp* in *Lactococcus lactis* or *Enterobacter facium* did not enhance biofilm formation, suggesting that Esp stimulates biofilm formation by *E. faecalis* through interaction with another component of *E. faecalis* such as a surface protein or an exopolymer (Tendolkar *et al.*, 2005). In addition, an *esp* mutant was found to have decreased cell surface hydrophobicity compared to its isogenic wild type (Tendolkar *et al.*, 2005); a phenomenon which has also been observed for other members of the Bap family. The connection between expression of Bap-like proteins and increased cell surface hydrophobicity is somewhat surprising since a high serine/threonine content and a low theoretical pI of these proteins suggest that they would be highly charged and soluble in an aqueous environment. This apparent paradox might be related to an interaction of Bap-like proteins with other matrix components such as polysaccharides.

LapA (large adhesion protein A) was first identified by Espinosa-Urgel *et al.* (2000) in a study aimed at determining factors of *P. putida* which are involved in adhesion to corn seed. Among a number of *mus* (mutants unattached to seed) mutants one (*mus*-24) displayed a very severe corn adhesion defect. This mutant was also tested for adhesion to abiotic surfaces and was found to be severely defective in adhesion to polystyrene, polypropylene and borosilicate glass in both minimal and rich media. Co-inoculation of the wild type and the *mus*-24 strain in part rescued the adhesion defect of the *mus*-24 strain, suggesting that the adhesion factor was secreted or that coaggregation with the wild type occurred. A gene almost identical to the *mus*-24 gene was identified in a transposon mutant screen for attachment defective mutants in *P. fluorescens* by O'Toole and Kolter (1998a). The *P. fluorescens* protein was further characterized by Hinsa *et al.* (2003) who designated the protein LapA. The LapA protein was reported to have sequence similarities to the CshA adhesin of the oral bacterium *Streptococcus gordonii* (Hinsa *et al.*, 2003). CshA has been shown to be essential for colonization of the oral cavity and to participate in coaggregation of *S. gordonii* with other oral bacteria (McNab *et al.*, 1996). LapA is transported to the bacterial surface via an ABC transport system which is encoded by the *lapEBC* genes, and is analogous to the type 1 transporter associated with transport of the BapA protein of *S. enterica*. The LapE protein has been identified in earlier work by Buell and Anderson (1992) referred to as the AggA protein, and was shown to be necessary for adhesion of *P. putida* to plant roots. Since a *lapE* mutant does not display surface located LapA it seems likely that the phenotypes described by Buell and Anderson (1992) for the *aggA* mutant also applies to a *lapA* mutant. Further work indicated that the plant receptor might consist of carbohydrate moieties (Buell *et al.*, 1993) suggesting that LapA might also facilitate adhesion to carbohydrate residues. The involvement of LapA in adhesion to both abiotic

and biotic surfaces suggests a function as a general adhesion. So far, no conditions that rescue the biofilm formation defect of a *lapA* mutant have been reported, suggesting an important role in biofilm formation of *P. putida* and *P. fluorescens* under diverse conditions. Our unpublished experiments with *P. putida* suggest that LapA not only acts as a surface adhesin but also plays a role during the later stages of biofilm formation as a cell-to-cell adhesin. The GGDEF and EAL domain containing protein LapD appears to be necessary for the activity of the LapA protein, possibly through a mechanism involving transport of LapA to the cell surface (Hinsa and O'Toole, 2006).

The Bap-type protein of *Burkholderia cenocepacia*, AdhA, was identified as an adhesin mediating binding to cytokeratin 13 filaments, which are expressed on the apical surface of injured tracheobronchial epithelial cells (Urban *et al.*, 2005). It was found that in conjunction with cable pili, the AdhA protein was required for strong binding to the epithelial cells and for migration across the epithelium surface. Huber *et al.* (2002) identified mutants in *B. cenocepacia* that were impaired in biofilm formation in both microtiter trays and flow-chambers. These mutants (termed m13 and m15) had insertions in a gene that showed homology to the Bap protein of *S. aureus*, and surface protein extracts from them showed that they were missing a 22 kDa protein. The mutants obtained by Huber *et al.* (2002) were shown to have decreased surface hydrophobicity compared to that of the wild type. However, because the Huber *et al.* (2002) report does not contain sequence information no conclusive correlation between the studies in the two *B. cenocephacia* strains can be made.

In Gram-positive bacteria a large group of proteins termed MSCRAMM proteins (microbial surface components that recognize adhesive matrix molecules) has been described (Patti *et al.*, 1994). The large family of MSCRAMM proteins share many of the characteristics of the Bap-type protein family, but the functions have mostly been demonstrated in relation to adhesion to host factors such as fibronectin-, fibrinogen-, collagen-, and heparin-related polysaccharides, although this does not rule out a function in biofilm matrices as well.

Extracellular DNA as matrix component in biofilms

Because most, if not all, bacterial populations are accompanied by extracellular DNA (e.g. Lorenz and Wackernagel, 1994), and because most bacterial species bind to DNA (e.g. Dubnau, 1999), it appears that extracellular DNA may serve as a cell-to-cell interconnecting compound in many different biofilms. On top of a basal level of DNA release it appears that many bacteria, especially those that are able to develop natural competence, possess a specific DNA-release program. A correlation between DNA release and competence development has been established in many different bacteria including *Streptococcus pneumoniae* (Steinmoen *et al.*, 2002), *Bacillus subtilis* (Lorenz *et al.*, 1991), *Acinetobacter calcoaceticus* (Palmen and Hellingwerf, 1995), *Neisseria gonorrhoeae* (Dillard and Seifert, 2001), and *Pseudomonas stutzeri* (Stewart *et al.*, 1983). In all these cases DNA release and competence development was shown to occur in late-log phase cultures, and in some of the cases it has been documented that competence development is regulated through a quorum-sensing mechanism (e.g. Pestova *et al.*, 1996; Magnuson *et al.*, 1994). Since many kinds of polymers can function as biofilm matrix material, it is difficult to conceive that bacteria should release large amounts of costly information material solely with the purpose of stabilizing biofilms.

It is possible, that bacteria release DNA both in order to exchange genetic material, and in order to form and stabilize biofilms. The relatively long-lasting physical proximity of bacteria in biofilms enable the constituent cells to establish long term relationships with each other, and evidence has been presented that biofilms are optimal environments for transformation-based gene transfer (e.g. Li *et al.*, 2001; Wang *et al.*, 2002; Hendrickx *et al.*, 2003).

In the present chapter we limit the description of extracellular DNA in biofilms to *P. aeruginosa* as an example from the Proteobacteria and *Streptococcus* species as examples from the Gram-positive bacteria.

Extracellular DNA and biofilm formation by *P. aeruginosa*

Evidence for a role of extracellular DNA as cell-to-cell interconnecting compound in *P. aeruginosa* biofilms has been presented both for the *P. aeruginosa* PAO1 reference strain and for clinical *P. aeruginosa* isolates (Whitchurch *et al.*, 2002; Nemoto *et al.*, 2003). *P. aeruginosa* PAO1 biofilm formation in the wells of microtiter plates was attenuated by the presence of DNase I, and biofilm formation by *P. aeruginosa* PAO1 in flow-chambers was almost absent when the flow-chambers were perfused with medium containing DNase I (Whitchurch *et al.*, 2002). In addition, young *P. aeruginosa* PAO1 biofilms, which had been grown in flow-chambers perfused with DNase-free medium, were dispersed rapidly after addition of DNase I to the flowing medium, whereas older *P. aeruginosa* PAO1 biofilms were not dispersed by DNase I treatment, suggesting that other components than extracellular DNA stabilizes older *P. aeruginosa* PAO1 biofilms (Whitchurch *et al.*, 2002). Matsukawa and Greenberg (2004) investigated the composition of the extracellular matrix of mature *P. aeruginosa* PAO1 biofilms, and found that extracellular DNA by far was the most abundant polymer, although exopolysaccharide encoded by the *psl* genes appeared to be the most critical structural matrix component. In contrast to the finding that extracellular DNA is not the primary cell-to-cell interconnecting compound in mature *P. aeruginosa* PAO1 biofilms, Nemoto *et al.* (2003) found that mature biofilms formed by four different clinical *P. aeruginosa* isolates could be dispersed by DNase treatment, suggesting that extracellular DNA is the critical matrix component in mature biofilms formed by these *P. aeruginosa* strains. Long before biofilms became a central research area Murakawa (1973a,b) conducted a study to characterize extracellular "slime" produced by *P. aeruginosa*. The chemical composition of slimes from 20 clinical *P. aeruginosa* isolates was investigated, and it was found that slimes from 18 strains consisted primarily of DNA, while two strains with a mucoid phenotype produced slimes composed primarily of polyuronic acid (which most likely was alginate). Figure 4.3 visualizes the extracellular DNA matrix in a flow-chamber-grown *P. aeruginosa* PAO1 biofilm. Evidence has been presented that *P. aeruginosa* is capable of producing an extracellular DNase (Allesen-Holm *et al.*, 2006) which might have a role during biofilm dispersal processes.

PCR and Southern analysis have suggested that the extracellular DNA released from *P. aeruginosa* in biofilms and planktonic cultures is similar to whole-genome DNA (Steinberger and Holden, 2005; Allesen-Holm *et al.*, 2006). In agreement, it has been shown that different chromosomal genes, including *his*$^+$, *leu*$^+$, and *trp*$^+$, could be transferred by transformation of $CaCl_2$-treated *P. aeruginosa* cells with extracellular DNA at the

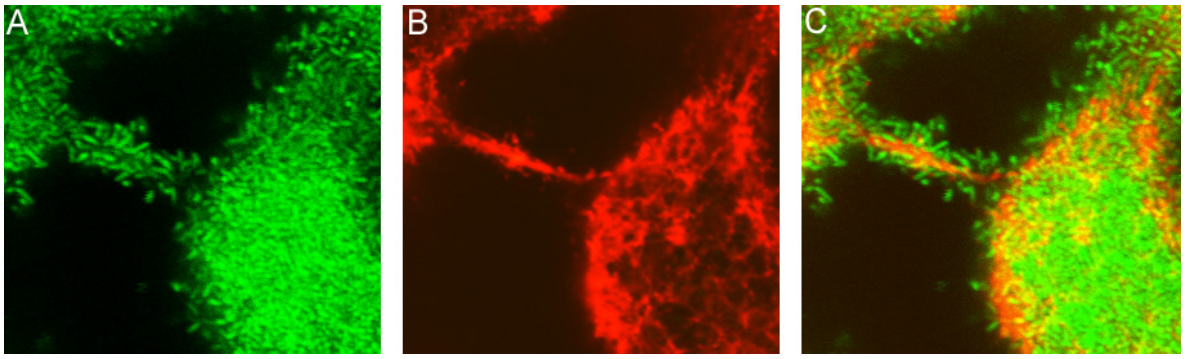

Figure 4.3 Horizontal confocal laser scanning microscope sections in a 2-day-old DDAO-stained biofilm formed by Gfp-tagged *P. aeruginosa* PAO1. The images show the fluorescent bacteria (A), the fluorescent extracellular DNA (B), and an overlay of the two (C). Reproduced from Mol. Microbiol. 59:1114–1128 with permission from Blackwell Publishing.

same frequencies as when transformation was done with an equivalent amount of purified intracellular DNA (Hara *et al.*, 1981; Muto and Goto, 1986).

A basal level of extracellular DNA present in *P. aeruginosa* PAO1 biofilms is evidently generated via a pathway which is not linked to quorum-sensing, whereas the generation of large amounts of extracellular DNA in *P. aeruginosa* biofilms evidently depends on the *las*, *rhl* and *pqs* quorum sensing systems (Allesen-Holm *et al.*, 2006). The increased level of extracellular DNA in *P. aeruginosa* wild-type biofilms in comparison to *P. aeruginosa lasIrhlI* biofilms appears to be linked to quorum-sensing via a mechanism that results in lysis of a small subpopulation of the cells (Allesen-Holm *et al.*, 2006). In support of a role of quorum-sensing in cell lysis, D'Argenio *et al.* (2002) reported that mutants which overproduce the *Pseudomonas* quinolone signal (PQS) displayed high levels of autolysis, whereas mutants which could not produce PQS did not show autolysis. In addition, Heurlier *et al.* (2005) presented evidence that *P. aeruginosa* quorum-sensing mutants, unlike the wild type, did not undergo cell lysis in stationary phase cultures. Quinolone compounds have previously been shown to induce prophages in bacteria (Phillips *et al.*, 1987; Froshauer *et al.*, 1996), and recent studies by Webb *et al.* (2003) and Hentzer *et al.* (2004) have suggested that quorum-sensing regulated DNA release might be linked to phage induction in biofilms. In support of a role of phage-mediated cell lysis in DNA release a *P. aeruginosa fliMpilA* mutant, which was reported not to undergo phage-mediated cell lysis (Webb *et al.*, 2003), showed a defect in DNA release (Allesen-Holm *et al.*, 2006). However, membrane vesicles produced by *P. aeruginosa* might also have a role in DNA release. *P. aeruginosa* releases membrane vesicles which have bacteriolytic effects and contain DNA (Kadurugamuwa and Beveridge, 1996; Renelli *et al.*, 2004). Extracellular DNA might be released either from vesicles that eventually lyse, or through the bacteriolytic activity of the vesicles which might lyse a small subpopulation of the *P. aeruginosa* cells. Recently it was shown that PQS is necessary for vesicle formation in *P. aeruginosa* (Mashburn and Whiteley, 2005), and evidence was presented that type IV pili and flagella are necessary for quorum sensing in *P. aeruginosa* (Hassett, 2005). The involvement of PQS, type IV pili, and flagella in DNA release, therefore, could be consistent with a role of vesicles in the generation of extracellular DNA in *P. aeruginosa* biofilms.

The extracellular DNA appears to be organized in distinct patterns in *P. aeruginosa* biofilms (Allesen-Holm *et al.*, 2006). In 4-day-old flow-chamber-grown *P. aeruginosa* biofilms, which contain mushroom-shaped structures, the extracellular DNA was located primarily in the stalk-portion of the mushroom-shaped structures with the highest concentration in the outer parts of the stalks forming a border between the stalk-subpopulation and the cap-subpopulation (Allesen-Holm *et al.*, 2006). The finding that biofilms formed by wild-type *P. aeruginosa* contained the highest concentration of extracellular DNA in the stalk-portion of the mushroom-shaped structures is in agreement with a study showing that the expression of *lasI* and *rhlI* in *P. aeruginosa* biofilms was highest in the portion of the biofilm closest to the substratum (DeKievit *et al.*, 2001). In addition it was shown that synthesis of rhamnolipid, an established quorum-sensing regulated process (Ochsner and Reiser, 1995; Pearson *et al.*, 1997), occurs primarily in the stalks of the mushroom-shaped structures in *P. aeruginosa* biofilms (Lequette and Greenberg, 2005). Evidence has been presented that the formation of the mushroom-shaped structures in glucose-grown *P. aeruginosa* biofilms occurs in a sequential process involving a non-motile bacterial subpopulation that forms the stalks by growth in certain foci of the biofilm, and a migrating bacterial subpopulation which subsequently forms the mushroom caps via a process that requires type IV pili (Klausen *et al.*, 2003a). It is currently not understood how the migration of the motile cells is coordinated so that they form mushroom caps. However, because type IV pili bind to DNA (Aas *et al.*, 2002; van Schaik *et al.*, 2005), it is tempting to speculate that the high concentration of extracellular DNA on the outer parts of the mushroom stalks might cause accumulation of the migrating bacteria, which in combination with bacterial growth, might result in the formation of the mushroom caps. In agreement with this suggestion, type IV pili-mediated migration of Myxobacteria during fruiting body formation has been shown to depend on the presence of exopolymer material (Lu *et al.*, 2005). The extracellular DNA in *P. aeruginosa* biofilms appears to have a stabilizing effect, as mature *P. aeruginosa* PAO1 biofilms which were pre-treated with DNase I were more susceptible to SDS treatment than biofilms which were not pre-treated with DNase I (Allesen-Holm *et al.*, 2006).

P. aeruginosa colonizes the lungs of cystic fibrosis (CF) patients and is a major cause of lung deterioration, health decline, and death of these patients (Høiby, 2002). Several studies have shown that *P. aeruginosa* forms biofilms in the CF lung (e.g. Lam *et al.*, 1980; Baltimore *et al.*, 1989; Worlitzsch *et al.*, 2002; Høiby, 2002), and the biofilm mode of growth is considered the major reason that these bacteria can not be eradicated by host defenses or antibiotic treatment (Costerton *et al.*, 1999). CF lungs evidently contain large amounts of extracellular DNA from necrotized neutrophils (Lethem *et al.*, 1990), and evidence has been presented that extracellular actin-DNA filaments can provide a matrix for biofilm formation by *P. aeruginosa* (Walker *et al.*, 2005). In another study the presence of extracellular DNA was shown to be important for *P. aeruginosa* biofilm formation in artificial CF sputum medium (Sriramulu *et al.*, 2005). In addition to a role of extracellular DNA, it was reported that biofilm formation in artificial CF sputum medium depended on the presence of amino acids (Sriramulu *et al.*, 2005). Evidence has been presented that *P. aeruginosa*, in part due to the presence of a high level of aromatic amino acids, produces large amounts of PQS when it is present in CF lungs (Collier *et al.*, 2002; Palmer *et al.*, 2005). Because PQS evidently plays a role in DNA release from *P. aeruginosa* (Allesen-Holm *et al.*, 2006),

PQS-mediated release of DNA from the bacteria might play a role in biofilm formation in the CF lung. In further support of this possibility, the amino acid content of CF sputum has been shown to correlate with the severity of the disease (Thomas *et al.*, 2000).

Extracellular DNA and biofilm formation by streptococci

Evidence that extracellular DNA plays a role in biofilm formation by streptococci is accumulating steadily. Competence mutants of *S. mutans* and *S. gordonii* were shown to be attenuated in biofilm formation (Loo *et al.*, 2000; Li *et al.*, 2002; Yoshida *et al.*, 2002), and because these competence mutants were also deficient in generating extracellular DNA, it is possible that the biofilm formation defect was caused by a lack of extracellular DNA. Accordingly, the presence of DNase I was subsequently shown to attenuate biofilm formation by *S. mutans* and *S. intermedius* wild-type strains in the wells of microtiter plates (Petersen *et al.*, 2004; 2005). Addition of exogenous quorum-sensing signal molecules to *S. intermedius* cultures was shown to promote biofilm formation, and simultaneous treatment with DNase I was shown to reverse the effect, suggesting that extracellular DNA was responsible for the increase in biofilm formation upon addition of signal molecules (Petersen *et al.*, 2004). Among the genes regulated by quorum-sensing in streptococci are those required for DNA binding and uptake of extracellular DNA. Evidence has been presented, that proteins in streptococci which are necessary for binding and uptake of extracellular DNA play a role in biofilm formation. Petersen *et al.* (2005) reported that a *comGB* mutant of *S. mutans*, which is deficient in DNA binding but unaffected in quorum-sensing signaling, showed reduced biofilm formation. In the presence of DNase I, biofilm formation by the *S. mutans* wild type was reduced to a level similar to that displayed by the *comGB* mutant. The *comGB* mutant was not impaired in DNA-release as growth in the presence of quorum-sensing signaling molecules promoted DNA-release from both the wild type and the *comGB* mutant. The addition of exogenous quorum-sensing signaling molecules to *S. mutans* wild-type cultures was shown to promote DNA-release and biofilm formation, and the simultaneous addition of DNase I reversed the effect, emphasizing the importance of extracellular DNA in the biofilm formation process (Petersen *et al.*, 2005). Moreover, it was shown that addition of exogenous quorum-sensing signaling molecules to cultures of *S. mutans comX*, *comE*, and *comD* competence mutants did not promote DNA-release and biofilm formation. Evidence has been presented that *S. pneumoniae* is capable of producing an extracellular DNase (Moscoso and Claverys, 2004) which might have a role during biofilm dispersal processes.

The extracellular DNA in streptococcal populations appears to be generated via lysis of a subpopulation of the cells (e.g. Steinmoen *et al.*, 2002; 2003; Moscoso and Claverys, 2004; Shibata *et al.*, 2005), and should therefore be similar to whole genome DNA. Release of DNA in *S. pneumoniae* populations was shown to involve cell lysis via the cell-wall hydrolases LytA, LytC, and CbpD (Steinmoen *et al.*, 2003; Moscoso and Claverys, 2004; Guiral *et al.*, 2005). Evidence has been provided that competent *S. pneumoniae* cells trigger lysis of *S. pneumoniae* sibling cells that are non-competent because they respond slower to the quorum-sensing signaling molecules (Steinmoen *et al.*, 2003; Moscoso and Claverys, 2004; Guiral *et al.*, 2005). The phenomenon evidently involves a system consisting of a bacteriocin (CibAB), its immunity factor (CibC), and the cell wall hydrolases (Guiral *et al.*, 2005).

Competent cells are immune to the bacteriocin, presumably because they also produce the immunity factor, but the bacteriocin induces lysis of non-competent cells via a process that depends on the cell-wall hydrolases. A similar bacteriocin-based system appears to operate during DNA release from *S. mutans* populations (Kreth *et al.*, 2005; van der Ploeg, 2005) and from *S. sanguis* populations (Schlegel and Slade, 1973).

Because biofilms most often contain numerous microenvironments, the streptococcal DNA-release mechanism described above will most likely lead to a stratified distribution of extracellular DNA in biofilms formed by streptococci. However, the investigation of structural biofilm development by the streptococci is not as advanced as in the case of *P. aeruginosa*, and at present the spatial organization of the extracellular DNA in streptococcal biofilms has not been investigated.

Competence-triggered DNA-release from streptococci has been proposed to ensure coordination in time and space between DNA-release and uptake, thus favoring genetic exchange (e.g. Steinmoen *et al.*, 2002; 2003). However, the finding that DNA-release in *S. pneumoniae* cultures continued a long time after competence had disappeared suggested that genetic exchange is not the only purpose of competence-triggered cell lysis (Moscoso and Claverys, 2004). Competence-triggered lysis of streptococcal cells could be important for the release of virulence factors such as pneumolysin, (lipo-) teichoic acid, and DNA (Guiral *et al.*, 2005). Because streptococci often are involved in biofilm-related infections such as those occurring in middle ears or lungs (e.g. *S. pneumoniae*) or on teeth (e.g. *S. mutans*), it is possible that the extracellular DNA plays a role in stabilizing medically relevant streptococcal biofilms. In agreement with this possibility the *comB*, *comD*, *lytA*, and *cbpD* genes have all been implicated in the virulence of *S. pneumoniae* (Jedrzejas, 2001; Bartilson *et al.*, 2001; Lau *et al.*, 2001; Hava *et al.*, 2003).

Concluding remarks

The extracellular matrix is arguably the most critical component of biofilms as it constitutes the framework that holds the component cells together. Indeed, it may be argued that the difference between a planktonic bacterium and a biofilm bacterium basically is that the biofilm bacterium has upregulated its adhesiveness and produces one or more biofilm matrix components. On top of that of course, the micro-environmental conditions prevailing in the different parts of a biofilm during the different stages of biofilm development leads to the expression of distinct sets of genes in time and space.

Many different compounds may function as extracellular biofilm matrix component. The emerging picture is that almost anything that can interconnect bacteria may function as a matrix component. Recent research in the field has provided some surprises. For example it has been found that mating pili and extracellular DNA can function as biofilm matrix components. By promoting biofilm formation, mating pili and extracellular DNA also create optimal environments for gene transfer via conjugation or transformation. In this way efficient gene transfer may be both a consequence and a cause of biofilm development.

A single bacterial species can produce several different biofilm matrix components. Usually not all of the biofilm matrix components are expressed during biofilm formation in a particular environment, but it is anticipated that the capacity of bacteria to produce dif-

ferent biofilm matrix components allows colonization of different niches through different biofilm development pathways.

Although the matrix components used for biofilm development are diverse and vary amongst bacterial species, and in response to environmental cues, there may be common features underlying these factors. Recent work indicates that one common denominator to bacterial adhesiveness and biofilm matrix production may be regulatory proteins which contain GGDEF and/or EAL domains. Through diguanylate cyclase or phosphodiesterase activity proteins with GGDEF or EAL domains control intracellular levels of c-di-GMP which acts as a second messenger and affects matrix production and the adhesiveness of the bacteria. It appears that in many cases, exemplified in this chapter by *S. enterica*, *P. fluorescens*, *P. putida*, and *P. aeruginosa*, GGDEF/EAL domain proteins regulate production (or transport) of both polysaccharide and protein components of the biofilm matrix.

Biofilms may be very dynamic and contain migrating bacterial subpopulations, and it appears that the biofilm matrix may serve as a framework that the bacteria can migrate on. Evidence is emerging that exopolymer material is necessary for coordinated bacterial migration during structural development in *P. aeruginosa* biofilms and in myxobacterial biofilms.

Continued research in the field will improve our understanding of the composition of extracellular matrices in biofilms formed by particular species under particular conditions, and will provide knowledge about the regulation of bacterial adhesiveness and matrix production and the transition between planktonic and biofilm lifestyles. Interference with the production of biofilm matrix components, or with the physical integrity of the biofilm matrix, are obvious therapeutic strategies for combating biofilm-based persistent infections.

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Paper 3

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Tolerance to the Antimicrobial Peptide Colistin in *Pseudomonas aeruginosa* Biofilms is Linked to Metabolically Active Cells, and Depends on the *pmr* and *mexAB-oprM* genes.

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Bacteria living as biofilm are frequently reported to exhibit inherent tolerance to antimicrobial compounds, and might therefore contribute to the persistence of infections. Antimicrobial peptides are attracting increasing interest as new potential antimicrobial therapeutics, however, little is known about potential mechanisms, which might contribute to resistance or tolerance development towards these compounds in biofilms. Here we provide evidence that a spatially distinct subpopulation of metabolically active cells in *Pseudomonas aeruginosa* biofilms is able to develop tolerance to the antimicrobial peptide colistin. On the contrary, biofilm cells exhibiting low metabolic activity were killed by colistin. We demonstrate that the subpopulation of metabolically active cells is able to adapt to colistin by inducing a specific adaptation mechanism mediated by the *pmr*-operon, as well as an unspecific adaptation mechanism mediated by the *mexAB-oprM*-genes. Mutants defective in either *pmr*-mediated LPS-modification or in *mexAB-oprM*-mediated antimicrobial efflux were not able to develop a tolerant subpopulation in biofilms. In contrast to the observed pattern of colistin-mediated killing in biofilms, conventional antimicrobial compounds such as ciprofloxacin and tetracycline were found to specifically kill the subpopulation of metabolically active biofilm cells, whereas the subpopulation exhibiting low metabolic activity survived the treatment. Consequently, targeting the two physiologically distinct subpopulations by combined antimicrobial treatment with either ciprofloxacin and colistin or tetracycline and colistin almost completely eradicated all biofilm cells.

Introduction

In nature, bacteria are constantly exposed to a variety of small bioactive natural products, also referred to as secondary metabolites. Secondary metabolites are produced by virtually all living organisms on earth. The ecological role of these chemical compounds is still a matter of debate, and might range from waste product removal, to signalling or defence. Some of these compounds can exhibit bacteriocidal or bacteriostatic activities and are now frequently used in medical settings as antibiotics to treat bacterial infections. However, bacteria have evolved different strategies to sense, respond and adapt to these small chemical compounds. In the present study we investigate the spatiotemporal-dependent effects and mechanisms taking place in an established microbial community of *P. aeruginosa* upon exposure to the antimicrobial peptide colistin.

P. aeruginosa is a metabolically versatile Gram-negative bacterium, which can be found as natural inhabitant of terrestrial and aquatic environments as well as associated with animals and plants (Ramos, 2004). In humans it can frequently cause life-threatening infections under conditions where the host is injured, the immune system is compromised, or in individuals who are afflicted with cystic fibrosis (CF) (Bodey *et al*., 1983; Lyczak *et al*., 2002).

It is assumed that the majority of bacteria in nature are living in spatially distinct communities, also referred to as biofilms (Davey and O'Toole, 2000). A number of human infectious diseases have been associated with the bacterial biofilm mode of living (Høiby *et al*., 2001; Parsek and Singh, 2003; Hall-Stoodley *et al*., 2006). It is believed that these infections are difficult to treat with conventional antimicrobial therapy, because the causative bacteria reside in biofilms and are intrinsically tolerant to

antimicrobial attack (Costerton *et al*., 1999; Stewart and Costerton, 2001; Donlan and Costerton, 2002). Several reasons have been suggested to explain this phenomenon, such as i) poor penetration of the antimicrobial compound, ii) slow growth, or iii) the presence of specialized persister cells or phenotypic variants (Mah and O'Toole, 2001; Lewis, 2001; Stewart, 2002; Drenkard, 2003; Fux *et al*., 2005). However, recent observations indicate that, dependent on the antimicrobial compound used, biofilm cells do not necessarily exhibit higher tolerance than planktonic cells (Brooun *et al*., 2000, Spoering and Lewis, 2001). Therefore detailed knowledge on biofilm physiology, and on bacterial adaptation responses in biofilms towards particular antimicrobial compounds is required to design new treatment strategies against persistent infections.

Antimicrobial peptides (AMPs) are attracting increasing interest as new potential antimicrobial therapeutics, because i) resistance to antimicrobial peptides has rarely been observed (Boman, 1995; Hancock, 2001; Peschel and Sahl, 2006) and moreover ii) due to their ability to modulate innate immune response (Boman, 1995; Hancock and Sahl, 2006). Antimicrobial peptides are a structurally diverse group of molecules, which share cationic and amphipathic properties and are found throughout all domains of life (Boman, 1995; Hancock and Chapple, 1999; Zasloff, 2002; Peschel and Sahl, 2006). It is generally assumed that the primary target of AMPs is the microbial membrane. However, there is increasing evidence that antimicrobial peptides can also have intracellular targets (Otvos, 2005; Brogden, 2005). It is still somewhat of an enigma why highly effective resistance mechanisms towards AMPs have not evolved so far, since microorganisms have been exposed to these peptides presumably for millions of years (Peschel and Sahl, 2006).

The antimicrobial peptide colistin (Polymyxin E) has recently received raising attention due to its significant effect on multi-drug resistant Gram-negative bacteria (Falagas and Kasiakou, 2005; Li *et al*., 2006). Colistin belongs to a group of peptide antibiotics, the polymyxins, which are synthesized in nature by strains of *Paenibacillus* spp., (e.g. *Paenibacillus polymyxa*) (Ainsworth *et al*., 1947; Benedict and Langlykke, 1947; Stansly *et al*., 1947; Suzuki *et al*., 1965; Storm *et al*., 1977). In clinical settings these compounds are administered for treatment of various infections caused by Gram-negative bacteria, e.g. sepsis, wound infections, urinary tract infection, pneumoniae, catheter-related infections and otitis media (Levin *et al*., 1999; Garnacho-Montero *et al*., 2003; Markou *et al*., 2003; Karabinis *et al*., 2004). However, the primary use of colistin is in treatment of lung infections caused by *P. aeruginosa* in patients afflicted with CF (Jensen *et al*., 1987; Johansen *et al.*, 2004; Littlewood *et al*., 1985; Littlewood *et al*., 2000). Tolerance-development to polymyxins (and other antimicrobial peptides) in Gram-negatives appears to be conditional and involves modifications of lipopolysaccharide (LPS) that decrease the overall negative net charge of the bacterial outer surface, and hence the interaction with AMPs. Among those modifications is the addition of aminoarabinose to negatively charged phosphate groups of lipid A (Helander *et al*., 1994; Ernst *et al*., 1999; Zhou *et al*., 2001; Trent *et al*., 2001). The modification is mediated by the gene products of the *pmrHFIJKLM*-operon (or homologues), present in Gram-negative bacteria such as *Salmonella typhimurium*, *P. aeruginosa* and *Escherichia coli* (Gunn *et al*., 1998; Trent *et al*., 2001; McPhee *et al*., 2003). In *P. aeruginosa* the expression of *pmrHFIJKLME* (PA3552-3559) can be induced under conditions of low Mg^{2+}, and in the presence of antimicrobial peptides (such as polymyxins) or polyamines (such as spermidine) (McPhee *et al*., 2003; Kwon and Lu, 2006). Two two-component systems, namely PhoPQ and PmrAB, have been identified to regulate the expression of *pmrHFIJKLME* under these environmental conditions (Ernst *et al*., 1999; Mcfarlane *et al*., 2000; McPhee *et al*., 2003; Moskowitz *et al*., 2004). However, the reported data suggested that other determinants might play a role in peptide-mediated tolerance-development as well (McPhee *et al*., 2003).

Haagensen *et al.* (2007) recently presented evidence that the *pmr*-operon is involved in the development of a colistin-tolerant subpopulation of cells in *P. aeruginosa* PAO1 flow-chamber-grown biofilms. In addition to colistin tolerance, the cells of this particular subpopulation were found to exhibit surface associated motility. However, whether there was a link between tolerance development to colistin and motility, or whether the apparent correlation was indirect was not clear. Here we provide evidence that development of tolerance to colistin in mature *P. aeruginosa* biofilms is dependent on cellular metabolic activity but independent of cellular motility. The metabolically active cells are able to adapt to colistin treatment by inducing a specific adaptation mechanism mediated by the *pmr*-operon, as well as an unspecific

adaptation mechanism mediated by *mexAB-oprM*. Moreover, we show that by specifically targeting physiologically distinct subpopulations present in a biofilm, it is possible to eradicate most of the cells in the biofilm.

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in table 1. For routine strain manipulations *P. aeruginosa* and *E. coli* strains were grown in LB medium at 37ºC. For batch culture experiments with *P. aeruginosa* AB minimal medium (Pamp and Tolker-Nielsen, 2007) supplemented with 1 μM $FeCl_3$ and 10 mM glucose was used. The clinical non-mucoid isolates *P. aeruginosa* CFSJ208 and CFSJ234 were isolated from sputum obtained from 23 and 25 years old patients respectively, who are afflicted with cystic fibrosis. Isolation was carried out using Pseudomonas isolation agar (PIA) and the strains verified using phenotypic analysis and PCR. Where appropriate, antibiotics were used for bacterial cultures at the following concentrations: for *P. aeruginosa*, gentamycin (Biochrome AG, Germany) at 30 μg/ml, streptomycin (Sigma, Germany) at 300 μg/ml, potassium tellurite (Sigma, Germany) at 150 μg/ml and carbenicillin (Sigma, Germany) at 200 μg/ml; for *E. coli*, ampicillin (Vepidan ApS, Denmark) at 100 μg/ml, chloramphenicol (Sigma, Germany) at 25 μg/ml, kanamycin (Sigma, Germany) at 50 μg/ml, and gentamycin at 15 μg/ml.

DNA manipulations

Plasmids and primers used in this study are listed in table 1. DNA restriction enzyme digestions and modifications were performed according to the manufacturer's instructions (Fermentas, Invitrogen). Plasmids were transformed using electroporation if not otherwise stated: for *P. aeruginosa*, 25 mF, 200 Ω, <5 ms, 2.5 kV; for *E. coli*, 25 mF, 400 Ω, <5 ms, 2.5 kV. *P. aeruginosa* strains were fluorescently tagged at an intergenic neutral chromosomal locus (25 bp downstream of the *glmS* gene) in miniTn7 constructs, as described previously (Lambertsen, *et al*., 2004). The insertion was verified by PCR using the primers Tn7-GlmS and Tn7-R109, as described previously (Lambertsen, *et al*., 2004). The strain *P. aeruginosa* PAO1 Δ*mexAB-oprM*::Gm^r was constructed as follows: A knockout fragment of Δ*mexAB-oprM* containing a gentamycin (Gm) resistance cassette was generated by PCR overlap extension essentially as described by Choi and Schweizer (2005); shortly, primers MexA-UpF-GW, MexA-UpR-Gm, OprM-DnF-Gm, OprM-DnR-GW were used to amplify chromosomal regions of *mexA* and *oprM* respectively, and primer Gm-F and Gm-R were used to amplify the Gm cassette using plasmid pPS856 (Hoang *et al*. 1998) as template. The PCR fragments were fused together and amplified with primers GW-attB1 and GW-attB2 incorporating the *att*B1 and *att*B2 recombination sites at either end of the knockout cassette. Using the Gateway cloning system (Invitrogen) the resulting knockout fragment was first transferred via BP reaction into pDONR221 generating entry plasmid pDONR211-mexA-oprM and subsequently transferred via LR reaction into pEX18ApGW generating the knockout plasmid pEX18ApmexA-oprM. The knockout fragment Δ*mexAB-oprM*::Gm^r was transferred into *P. aeruginosa* PAO1 by triparental mating using helper strain *E.coli* HB101 pRK600. The resulting double crossover in strain *P. aeruginosa* Δ*mexAB-oprM*::Gm^r was confirmed by PCR and the phenotype regarding sensitivity to conventional antimicrobial agents compared with the known phenotype of strain *P. aeruginosa* PAO200. The strains *P. aeruginosa* PAO1 Δ*mexPQ-oprE*::Gm^r, *P. aeruginosa* PAO1 Δ*mexGHI-oprD*::Gm^r, and *P. aeruginosa* PAO1 Δ*yegMNO-opmB*::Gm^r were constructed in exactly the same way, with the exception that the following gene-specific primers were used: MexP-UpF-GW + MexP-UpR-Gm and OprE-DnF-Gm + OprE-DnR-GW for the construction of the *mexPQ-oprE* mutant, MexG-UpF-GW + MexG-UpR-Gm and OprD-DnF-Gm + OprD-DnR-GW for the construction of the *mexGHI-oprD* mutant, and YegM-UpF-GW + YegM-UpR-Gm and OpmB-DnF-Gm + OpmB-DnR-GW for the construction of the *yegMNO-opmB* mutant.

Cultivation of biofilms

Biofilms were cultivated in flow-cells with individual channel dimensions of 1 x 4 x 40 mm, covered with a glass coverslip (Knittel Gläser, Germany) as substratum for biofilm formation. The biofilm flow-cell system was assembled and prepared as described elsewhere (Sternberg and Tolker-Nielsen, 2005). AB minimal medium (Pamp

Table 1. Strains, plasmids, and primers used in this study

Strain, plasmid or primer	Relevant characteristics or sequence[a]	Source or reference
P. aeruginosa		
PAO1	wild type	Holloway and Morgan, 1986; (J. S. Mattick)
PAO1 Gfp	*P. aeruginosa* PAO1 tagged with eGfp in a mini-Tn7 construct; Gm^r	Klausen *et al.*, 2003a
PAO1 *pilA* Gfp	*pilA* inactivated in *P. aeruginosa* PAO1 by allelic displacement with a tellurite resistance cassette using pTTN80; tagged with Gfp in a mini-Tn7 construct; Tel^r, Gm^r	Klausen *et al.*, 2003a
PAO1 *fliM* Gfp	*fliM* inactivated in *P. aeruginosa* PAO1 by allelic displacement with a tetracycline resistance cassette using pTTN61; tagged with eGfp in a mini-Tn7 construct; Tet^r, Gm^r	Klausen *et al.*, 2003a
CFSJ208	non-mucoid *P. aeruginosa* strain; isolated from sputum derived from a 23 years old patient afflicted with cystic fibrosis	This study
CFSJ208 Gfp	*P. aeruginosa* CFSJ208 tagged with eGfp in a mini-Tn7 construct; Gm^r	This study
CFSJ234	non-mucoid *P. aeruginosa* strain; isolated from sputum derived from a 25 years old patient afflicted with cystic fibrosis	This study
PAO1 *rrnB*P1-*gfp*	*P. aeruginosa* PAO1 tagged with the transcriptional reporter fusion *rrnB*P1-*gfp* in a mini-Tn7 construct; Gm^r	This study
PAO1 *rrnB*P1-*gfp*[AGA]	*P. aeruginosa* PAO1 tagged with the transcriptional reporter fusion *rrnB*P1-*gfp*[AGA] in a mini-Tn7 construct; Gm^r	This study
PAO1 P*pmrH-gfp*	*P. aeruginosa* PAO1 tagged with the transcriptional reporter fusion P*pmrH-gfp* in a mini-Tn7 construct; Gm^r	This study
PAO1 *pmrF* Gfp	*P. aeruginosa* PAO1 transposon mutant ID35399 - PA3553 (Washington Genome Center); tagged with eGfp in a mini-Tn7 construct; Gm^r	Haagensen, *et al.*, 2007
PAO1 Δ*mexAB-oprM*::Gm^r	*mexAB-oprM* inactivated in *P. aeruginosa* PAO1 by allelic displacement with a gentamycin resistance cassette using pEX18ApmexA-oprM; Gm^r	This study
PAO1 Δ*mexAB-oprM*::Gm^r Gfp	PAO1 Δ*mexAB-oprM*::Gm^r tagged with Gfp in a mini-Tn7 construct; Gm^r, Sm^r	This study
PAO1 Δ*mexPQ-oprE*::Gm^r	*mexPQ-oprE* inactivated in *P. aeruginosa* PAO1 by allelic displacement with a gentamycin resistance cassette using pEX18ApmexP-oprE; Gm^r	This study
PAO1 Δ*mexPQ-oprE*::Gm^r Gfp	PAO1 Δ*mexPQ-oprE*::Gm^r tagged with Gfp in a mini-Tn7 construct; Gm^r, Sm^r	This study
PAO1 Δ*mexGHI-oprD*::Gm^r	*mexGHI-oprD* inactivated in *P. aeruginosa* PAO1 by allelic displacement with a gentamycin resistance cassette using pEX18ApmexG-oprD; Gm^r	This study
PAO1 Δ*mexGHI-oprD*::Gm^r Gfp	PAO1 Δ*mexGHI-oprD*::Gm^r tagged with Gfp in a mini-Tn7 construct; Gm^r, Sm^r	This study
PAO1 Δ*yegMNO-opmB*::Gm^r	*yegMNO-opmB* inactivated in *P. aeruginosa* PAO1 by allelic displacement with a gentamycin resistance cassette using pEX18ApyegM-opmB; Gm^r	This study
PAO1 Δ*yegMNO-opmB*::Gm^r Gfp	PAO1 Δ*yegMNO-opmB*::Gm^r tagged with Gfp in a mini-Tn7 construct; Gm^r, Sm^r	This study
PAO1 p*mexA-gfp*	*P. aeruginosa* PAO1 harbouring plasmid p*mexA-gfp*; Amp^R	This study
PAO1 p*mexC-gfp*	*P. aeruginosa* PAO1 harbouring plasmid p*mexA-gfp*; Amp^R	This study
$PAO1^{(Schweizer)}$	wild type	Watson and Holloway, 1978; (H. P. Schweizer)
$PAO1^{(Schweizer)}$ Gfp	*P. aeruginosa* $PAO1^{(Schweizer)}$ tagged with eGfp in a mini-Tn7 construct; Gm^r	This study
PAO200	*P. aeruginosa* PAO1 Δ(*mexAB-oprM*)	Schweizer, 1998
PAO200 Gfp	PAO200 tagged with eGfp in a mini-Tn7 construct; Gm^r	This study
E. coli		
HB101	*recA thi pro leu hsdRM*, Sm^r; strain used for maintainance and proliferation of plasmids	Kessler, *et al.*, 1992
DH5α	F^-, φ80d*lacZ*Δ M15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1	Invitrogen
Plasmids		
pUX-BF13	mob^+ *ori*-R6K; helper plasmid providing the Tn7 transposition functions *in trans*; Amp^r	Bao *et al.*, 1991

Table 1. –*continued*

Strain, plasmid or primer	Relevant characteristics or sequence[a]	Source or reference
pBK-miniTn7(Gm^r)-gfp	delivery plasmid for miniTn7-$P_{A1/04/03}$-gfp; Amp^r, Gm^r	Koch *et al.*, 2001
pBK-miniTn7(Sm^r)-gfp	delivery plasmid for miniTn7-$P_{A1/04/03}$-gfp; Amp^r, Gm^r	Koch *et al.*, 2001
pBK-miniTn7-*rrnB*P1-*gfp*	delivery plasmid for miniTn7-*rrnB*P1-*gfp*; Amp^r, Gm^r	Lambertsen *et al.*, 2004
pBK-miniTn7-*rrnB*P1-*gfp*[AGA]	delivery plasmid for miniTn7-*rrnB*P1-*gfp*[AGA]; Amp^r, Gm^r	Lambertsen *et al.*, 2004
pBK-miniTn7-P*pmrH-gfp*	delivery plasmid for miniTn7-P*pmrH-gfp*; Amp^r, Gm^r	Haagensen *et al*, 2007
p*mexA-gfp*	pJK1 containing a *mexA-gfp* transcriptional fusion; Amp^r	De Kievit *et al.*, 2001
p*mexC-gfp*	pJK1 containing a *mexC-gfp* transcriptional fusion; Amp^r	De Kievit *et al.*, 2001
pEX18ApGW	Gateway compatible gene replacement vector ; Suc^s, Amp^r	Choi & Schweizer 2005
pPS856	0.83-kb blunt-ended SacI fragment from pUCGM ligated into the EcoRV site of pPS854; Amp^r, Gm^r	Hoang *et al.* 1998
pDONR221	Gateway donor vector; Km^r	Invitrogen
pDONR221mexA-oprM	*mexA-oprM* entry clone; Km^r, Gm^r	This study
pDONR221mexP-oprE	*mexP-oprE* entry clone; Km^r, Gm^r	This study
pDONR221mexG-oprD	*mexG-oprD* entry clone; Km^r, Gm^r	This study
pDONR221yegM-opmB	*yegM-opmB* entry clone; Km^r, Gm^r	This study
pEX18ApmexA-oprM	*mexA-oprM* knockout vector; Suc^s, Amp^r, Gm^r	This study
pEX18ApmexP-oprE	*mexP-oprE* knockout vector; Suc^s, Amp^r, Gm^r	This study
pEX18ApmexG-oprD	*mexG-oprD* knockout vector; Suc^s, Amp^r, Gm^r	This study
pEX18ApyegM-opmB	*yegM-opmB* knockout vector; Suc^s, Amp^r, Gm^r	This study
pRK600	*ori*-ColE1 RK2-*mob*$^+$RK2-*tra*$^+$; helper plasmid for conjugation; Cm^r	Kessler *et al.* 1992
Primers		
Tn7-GlmS	5'-AATCTGGCCAAGTCGGTGAC-3'	
Tn7-R109	5'-CAGCATAACTGGACTGATTTCAG-3'	
MexA-UpF-GW	5′-TACAAAAAAGCAGGCTGTAGTTCATTGGTTTGGCCGAG-3'	
MexA-UpR-Gm	5′-TCAGAGCGCTTTTGAAGCTAATTCGAACAGGCGCTTGAGGATGAT-3'	
OprM-DnF-Gm	5′-AGGAACTTCAAGATCCCCAATTCGAGACCGCGAAGAAGGAAGAT-3'	
OprM-DnR-GW	5′-TACAAGAAAGCTGGGTGACTTCGACAATTTCGGCAACC-3	
MexP-UpF-GW	5'-TACAAAAAAGCAGGCTGGATAGTCGTTCTTCCTGAGCA-3'	
MexP-UpR-Gm	5'-TCAGAGCGCTTTTGAAGCTAATTCGTGATGCGGATGAAGTGTCTGAG-3'	
OprE-DnF-Gm	5'-AGGAACTTCAAGATCCCCAATTCGGATGATGCCTTCAACG GCTATG-3'	
OprE-DnR-GW	5'-TACAAGAAAGCTGGGTAGAGTTCGGAACCGACTTATCG-3'	
MexG-UpF-GW	5'-TACAAAAAAGCAGGCTCACGTAGAGGTTTGCCATGCTT-3'	
MexG-UpR-Gm	5'-TCAGAGCGCTTTTGAAGCTAATTCGGTTGCTTTCGAGCGAGT TATCG-3'	
OprD-DnF-Gm	5'-AGGAACTTCAAGATCCCCAATTCGTCAACGTCATCGCGCTCTACAA-3'	
OprD-DnR-GW	5'-TACAAGAAAGCTGGGTATATCTACCTGCTGTCGCGGAT-3'	
YegM-UpF-GW	5'-TACAAAAAAGCAGGCTAGGAATACGCTCCAGTTCACCA-3'	
YegM-UpR-Gm	5'-TCAGAGCGCTTTTGAAGCTAATTCGTTCTTCAGGCTTAGAACGGAGG-3'	
OpmB-DnF-Gm	5'-AGGAACTTCAAGATCCCCAATTCGAGGACTACCTGGTGCAATTGAG-3'	
OpmB-DnR-GW	5'-TACAAGAAAGCTGGGTACACGTATTCACCTCCACATCC-3'	
Gm-F	5′-CGAATTAGCTTCAAAAGCGCTCTGA-3'	
Gm-R	5′-CGAATTGGGGATCTTGAAGTTCCT-3'	
GW-attB1	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3'	
GW-attB2	5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3'	

[a]The underlined sequence corresponds to the gene specific region.

and Tolker-Nielsen, 2007) supplemented with 0.3 mM glucose as carbon source was used as growth medium. Individual flow-cells were inoculated with 300 µl aliquots of overnight growth cultures of *P. aeruginosa*, which were adjusted to an optical density at 500 nm of 0.005. Overnight cultures of *P. aeruginosa* were grown in AB minimal medium supplemented with 30 mM glucose at 30ºC under vigorous shaking. To allow attachment of the bacterial cells to the substratum, flow-cells were left without flow for 1 hour after inoculation at 30ºC. Afterwards, a laminar flow with a mean flow velocity of 0.2 mm/s was achieved using a Watson Marlow 205S peristaltic pump. The *P. aeruginosa* strain containing plasmid p*mexA-gfp* was cultivated as biofilm without supplementation of carbenicillin to avoid any possible secondary effects during treatment with other antimicrobial compounds. Loss of plasmid during 4 days of biofilm growth was observed in a minor fraction of non-treated biofilms on average in less than 4 percent of cells, as examined by plating of dilutions of cells derived from biofilms on LB agar medium with and without carbenicillin.

Exposure of biofilms to antimicrobial and other compounds

Mature biofilms were exposed to the following compounds where indicated: 25 µg/ml colistin (*Colimycin*, colistin methanesulfonate; Lundbeck A/S, Denmark); 60 µg/ml ciprofloxacin (Bayer, Germany); 200 µg/ml tetracycline (Sigma, Germany); 30 µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Sigma, Germany). This was achieved by supplementing biofilm media with the required compounds at appropriate final concentrations and addition of the fluorescent dead-cell indicator propidium idodide (Sigma, Germany) at a final concentration of 0.3 µM. In non-treated control experiments 0.3 µM propidium iodide was added alone to the biofilm media. The colistin used here is colistin methanesulfonate, which is a compound undergoing hydrolysis in aqueous solutions to form the active compound colistin sulfate (CS) in a time-dependent manner (Bergen, *et al*., 2006). In control experiments CS (Sigma, Germany) was added in concentrations raging from 0.5 µg/ml to 8.0 µg/ml instead of colistin (i.e. colistin methanesulfonate). In these experiments the same spatial distribution of dead and alive cells was observed after 24 hours of exposure, compared to biofilms treated with 25 µg/ml colistin (colistin methanesulfonate). We used colistin (colistin methanesulfonate) in this study, since this is the actual compound, which is mainly used in medical settings for treatment of infections, in particular for treatment of *P. aeruginosa* lung infections in CF patients (Høiby *et al.*, 2005; Littlewood *et al*., 2000; Li *et al*., 2006). In some experiments 2.5 µM Syto 9 was added for counterstaining of living cells. To determine the survival rate upon antimicrobial treatment, cells were harvested from treated and non-treated biofilms and the number of surviving cells determined as follows: The bulk liquid was carefully removed from the flow-chambers and the biofilm cells recovered by pumping 1 ml 0.9% NaCl solution containing 50 µg glass beads (size ≤ 106 µm) (Sigma, Germany) rapidly back and forth through the flow-chamber until all cells were removed from the substratum as examined by microscopic inspection. The cell-suspension was mixed by vortexing and the number of living cells determined by plating dilutions of cells on LB agar medium and determination of the number of colony forming units (CFU) upon incubation.

Microscopy and image processing

Image acquisition was performed with a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Germany) equipped with an argon and a NeHe laser and detectors and filter sets for simultaneous monitoring of Gfp (excitation, 488 nm; emission, 517 nm) and propidium iodide (excitation, 543 nm; emission, 565 nm). Images were obtained using a 40x/1.3 Plan-Neofluar oil objective. Simulated multichannel cross-sections were generated using Imaris software package (Bitplane AG, Switzerland).

Results

When *P. aeruginosa* PAO1 is grown under continuous culture conditions in flow-chambers for four days with glucose as carbon source, it can form mature multicellular structures of mushroom-like shape (e.g. Fig 1A, and Klausen *et al*., 2003a). If this biofilm is exposed to 25 µg/ml colistin for 24 hours, only part of the biofilm is eradicated, as can be visualized by staining of dead cells with the fluorescent indicator propidium iodide: cells in the interior part close to the substratum are killed by colistin, whereas cells in the upper layer survive the treatment (Fig 1B, and Haagensen *et al*., 2007).

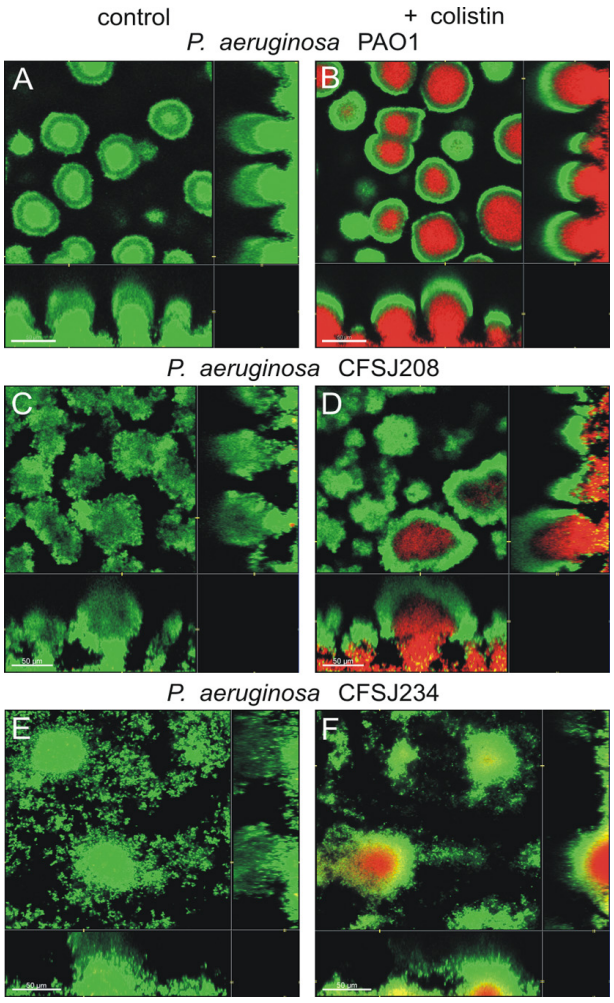

Fig. 1. Distribution of dead and live cells in colistin-treated *P. aeruginosa* biofilms. Biofilms of *P. aeruginosa* PAO1 Gfp (A+B), *P. aeruginosa* CFSJ208 Gfp (C+D) and *P. aeruginosa* CFSJ234 (E+F) were grown for 4 days and then continuously exposed to 25 µg/ml colistin and propidium iodide for 24 hours (B, D, F), or as control only to propidium iodide. The confocal laser scanning micrographs show a horizontal section with two flanking images representing sections in the xz and yz planes, respectively. Live cells appear green due to expression of Gfp (A, B, C, D) or due to staining with the fluorescent indicator dye Syto 9, specific for live cells (E+F). Dead cells appear red, due to staining with the dead-cell indicator propidium iodide.

Laboratory and clinical strains of P. aeruginosa exhibit a similar colistin tolerance phenotype in biofilms

Bacterial strains, which have been repeatedly passaged in the laboratory (as the strain used here), can under some conditions exhibit different phenotypes compared to environmental or clinical isolates (Velicier *et al*., 1998; Branda *et al*., 2001, Lee *et al*., 2005). In order to investigate, whether the PAO1 strain differed from clinical isolates with respect to colistin tolerance in biofilms, we investigated a number of *P. aeruginosa* strains, which had been isolated from sputum of young patients afflicted with cystic fibrosis, with respect to their sensitivity to colistin when grown as biofilm. The clinical isolates exhibited the same phenotype as the laboratory strain, namely a colistin-sensitive subpopulation close to the substratum and a colistin-tolerant subpopulation on top (two examples are shown in Fig. 1C-F). Because genetic variants evidently can arise at high frequency in some biofilms (Rainey and Travisano, 1998; Boles *et al*., 2004) it might be speculated that the cells, which survive the colistin treatment under our experimental conditions, are genetic variants inherently resistant to colistin. However, when tested on agar plates, cells harvested from colistin-treated biofilms formed by the laboratory strain or the clinical isolates did not exhibit any change in sensitivity to colistin compared to the cells used to initiate a biofilm (data not shown). This provides evidence that neither laboratory nor clinical strains develop inherent resistance to colistin during biofilm-growth in the flow-chamber system. Instead some cells exhibit phenotypic tolerance to colistin in biofilms and apparently only under circumstances when they are situated in the upper part of the biofilm.

Cellular migration per se is not involved in tolerance development towards colistin in mature biofilms

Studies employed to unravel the developmental stages and genetic determinants involved in formation of the mushroom-like structured *P. aeruginosa* biofilm have shown, that this biofilm is composed of at least two distinct subpopulations: a stalk-forming subpopulation situated at the substratum, and a cap-forming subpopulation on top (Haagensen *et al*., 2007; Klausen *et al*., 2003b; Pamp and Tolker-Nielsen, 2007). The studies demonstrated that the stalk-forming subpopulation is composed of non-motile cells, whereas the cap-forming subpopulation is formed by motile cells. In addition, the previous study on colistin tolerance in *P. aeruginosa* biofilms suggested, that the colistin-sensitive subpopulation spatially corresponds to the subpopulation of non-motile cells, whereas the colistin-tolerant subpopulation spatially corresponds to the subpopulation of motile cells (Haagensen *et al*., 2007). This suggests that cellular migration might

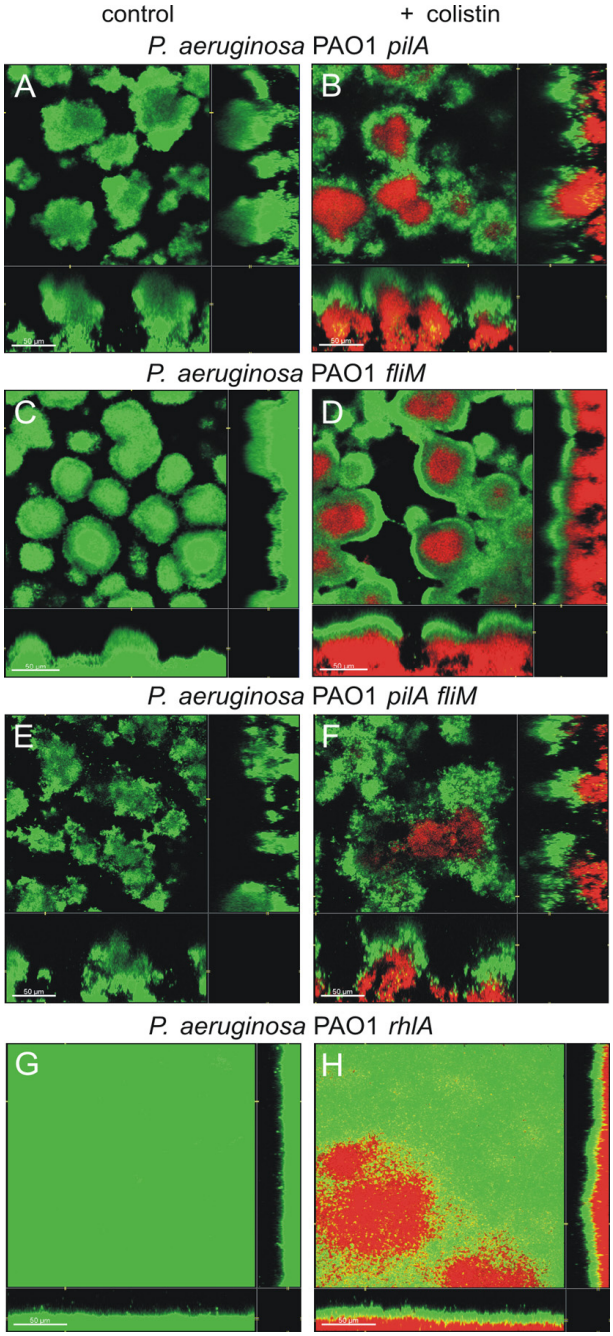

Fig. 2. Distribution of dead and live cells in colistin-treated biofilms formed by *P. aeruginosa* strains, which are impaired in cellular migration. Biofilms of *P. aeruginosa* PAO1 *pilA*::Tel^r Gfp (A+B), *P. aeruginosa* PAO1 *fliM*::Tet^r Gfp (C+D), *P. aeruginosa* PAO1 *pilA*::Tel^r *fliM*::Tet^r (E+F) and *P. aeruginosa* PAO1 *rhlA*::Gm^r (G+H) were grown for 4 days and then continuously exposed to 25 µg/ml colistin and propidium iodide for 24 hours (B, D, F, H), or as control to propidium iodide (A, C, E, G). The confocal laser scanning micrographs show a horizontal section with two flanking images representing sections in the xz and yz planes, respectively. Live cells appear green due to expression of Gfp and dead cells appear red, due to staining with the dead-cell indicator propidium iodide.

be involved in tolerance development to colistin. To test this hypothesis, we examined a number of motility-defective isogenic *P. aeruginosa* mutant strains with respect to tolerance development towards colistin in 4-day-grown biofilms. However all motility-defective mutants exhibited the same phenotype as the wild type: a sensitive subpopulation close to the substratum, and a tolerant subpopulation on top (Fig. 2, and data not shown). This suggests that, although bacterial migration is involved in structural development of the mushroom-like structured biofilm, it is not *per se* required for tolerance development to colistin in mature biofilms.

Metabolic/physiological activity is highest in cells situated in the upper layer of the biofilm

The observations described above suggested, that the physiology of cells situated in the upper layer of the flow-chamber-grown biofilm (cap-forming subpopulation) significantly differs from the physiology of the cells situated in the area closer to the substratum (stalk-forming subpopulation). To learn more about the physiology of *P. aeruginosa* cells grown in flow-chambers, we examined their metabolic/physiological activity by the use of a fluorescent reporter. Similar to as previously described (Andersen *et al.*, 1998; Sternberg *et al.*, 1999), we introduced the growth rate-dependent fluorescent reporter fusion *rrnB*P1-*gfp*[AGA] into our *P. aeruginosa* strain. In this case the *gfp*[AGA]-gene, encoding for a Gfp-protein with a short half-life, is placed under the transcriptional control of the ribosomal promotor *rrnB*P1. From cells of *P. aeruginosa rrnB*P1-*gfp*[AGA], which exhibit a high metabolic/physiological activity one can therefore expect a high fluorescent signal, whereas cells exhibiting a low metabolic/physiological activity would give no or a low fluorescent signal. As control we used *P. aeruginosa rrnB*P1-*gfp*, a strain expressing a stable version of Gfp, which should give an equal high fluorescent signal from all cells independent of the actual physiological state. When *P. aeruginosa rrnB*P1-*gfp*[AGA] was grown as a biofilm and CLSM images were acquired at intervals over a course of time of four days, a high fluorescent signal from cells situated in the upper layer could be detected at any time point (Fig. 3A). This suggests that the cells in the upper layer of the biofilm exhibited high metabolic/physiological activity. In contrast, cells located in the interior part of the multicellular structure exhibited a lower fluor-

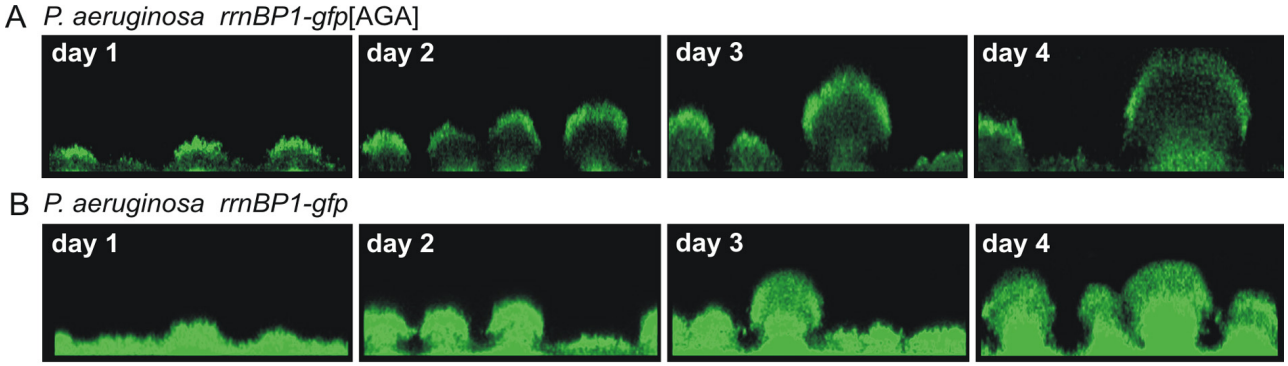

Fig. 3. Visualization of metabolic/physiological active cells in *P. aeruginosa* biofilms. *P. aeruginosa* PAO1 Tn7-*rrnB*P1-*gfp*[AGA] (A) and *P. aeruginosa* PAO1 Tn7-*rrnB*P1-*gfp* (B) were grown as biofilm and confocal laser scanning micrographs acquired every 24 hours for 4 days. Cells appear green either due expression of the unstable variant of the green fluorescent protein, Gfp[AGA] (A), or the stable version Gfp (B), both expressed under control of the ribosomal promotor *rrnB*P1. The images show vertical sections, respectively.

escent signal (Fig. 3A), indicating lower cellular metabolic/physiological activity. In some cases, a small fraction of cells exhibiting a high fluorescent signal could be observed in the stalk-part close to the substratum. The exact reason for this high signal is unknown at present. One reason could be that a high fluorescent signal is detected from this area because particularly here the cells are situated in very close proximity, resulting in a higher average number of cells compared to the rest of the structure, as can be seen using a higher magnification (data not shown; Fig. 1 in Haagensen *et al.*, 2007) As expected, an almost homogenous fluorescent signal from the entire biofilms of the control-strain *P. aeruginosa rrnB*P1-*gfp* was detected (Fig. 3B).

To get supporting evidence for the result involving the growth activity reporter, suggesting that specifically the biofilm cells in the upper layer of the biofilm are metabolically active, we chose to treat the biofilm with a compound that acts specifically on metabolically active cells. Ciprofloxacin was chosen, since it was shown to be significantly more effective on growing than non-growing planktonic cells of *P. aeruginosa*, and because penetration of this compound was shown not to be restricted in a colony biofilm of *P. aeruginosa* (Davey *et al*. 1988; Walters *et al*., 2003). We exposed a 4-day-grown biofilm of *P. aeruginosa rrnB*P1-*gfp*[AGA] to 60 μg/ml ciprofloxacin in the presence of the dead cell indicator propidium idodide, and followed the effect over 13 hours. As shown in Fig. 4A, ciprofloxacin killed only the cells in the upper layer of the biofilm, which exhibited high fluorescent signal. Ciprofloxacin killed neither the cells exhibiting a low fluorescent signal, nor the small fraction of cells close to the substratum, which exhibited a higher fluorescent signal. Also, prolonged exposure to ciprofloxacin did not lead to a killing-effect in the stalk-forming subpopulation of cells (data not shown). This provides evidence that only the cells in the upper layer of the flow-chamber-grown biofilm exhibit high metabolic/physiological activity.

Colistin kills preferably biofilm cells exhibiting low metabolic/physiological activity

To investigate if the killing-effect of colistin is confined to a distinct metabolic/physiological subpopulation of biofilm cells, a biofilm of the strain harbouring the growth activity reporter, *P. aeruginosa rrnB*P1-*gfp*[AGA], was grown for 4 days and subsequently exposed to 25 μg/ml colistin in the presence of the dead cell indicator propidium idodide. CLSM image acquisition showed that colistin specifically targeted the cells exhibiting low fluorescent signals, indicating that it preferably killed cells with low metabolic activity (Fig. 4B). In contrast, cells exhibiting high fluorescent signals survived the colistin treatment, indicating that biofilm cells with high metabolic activity exhibited tolerance towards colistin (Fig 4B).

Biofilm cells depleted of metabolic/physiological energy are sensitive towards colistin

The results described above suggested that an energy-driven active process might be involved in tolerance development towards colistin of the distinct upper subpopulation of cells in biofilms. To

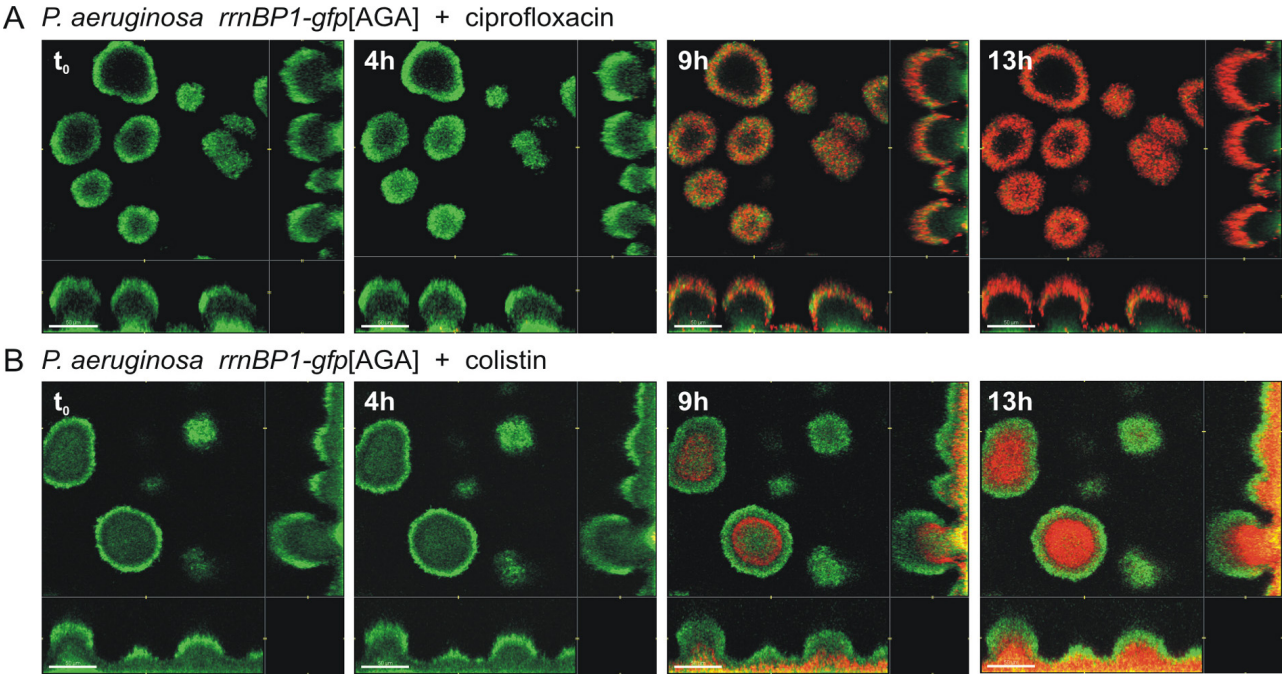

Fig. 4. Targeting distinct metabolic/physiological subpopulations in *P. aeruginosa* biofilms by ciprofloxacin and colistin. Biofilms of *P. aeruginosa* PAO1 Tn7-*rrnB*P1-*gfp*[AGA] were grown for 4 days and then continuously exposed to either 60 μg/ml ciprofloxacin and propidium iodide (A), or to 25 μg/ml colistin and propidium iodide (B). Confocal laser scanning micrographs were acquired at time point t_0 (prior to exposure) and 4, 9, and 13 hours subsequent to the beginning of treatment. The images show a horizontal section with two flanking images representing sections in the xz and yz planes, respectively. Metabolic active cells appear green due to expression of Gfp[AGA] under control of the ribosomal promotor *rrnB*P1 and dead cells appear red, due to staining with the dead-cell indicator propidium iodide.

obtain additional evidence for this proposition we chose to deplete the biofilm cells of metabolic energy using the uncoupler of oxidative phosphorylation CCCP. Biofilm cells treated with CCCP should not be able to develop tolerance towards colistin in the upper layer. When a 4-day-grown biofilm of *P. aeruginosa* was simultaneously exposed to 30 μg/ml CCCP and 25 μg/ml colistin (in the presence of propidium iodide), all cells in the biofilm were killed within 13 hours (Fig. 5A). However, no cells were killed in a 4-day-grown biofilm of *P. aeruginosa* when solely exposed to 30 μg/ml CCCP (and propidium iodide) (Fig. 5B). This finding substantiates the suggestion, that an active cellular process is required to develop tolerance towards colistin in *P. aeruginosa* biofilms.

Biofilm cells exhibiting high metabolic activity induce pmrHFIJKLME-expression upon colistin treatment and hence survive

It is known that *pmrHFIJKLME*-mediated LPS-modification is involved in tolerance towards colistin, and using the fluorescent promotor fusion P*pmrH-gfp* Haagensen and colleagues have recently shown, that this operon was induced in a 4-day-grown biofilm of *P. aeruginosa*, which had been exposed to colistin for 24 hours (Haagensen *et al.*, 2007). However, it was not investigated, when and where induction is initiated in the biofilm immediately upon exposure to colistin, and how this correlated to the appearance of dead cells in the interior part of the multicellular community in a spatiotemporal-dependent manner. In order to investigate spatiotemporal *pmrH*-induction and cell-death in more detail, we exposed a 4-day-grown biofilm of *P. aeruginosa* P*pmrH-gfp* to 25 μg/ml colistin in the presence of the dead-cell indicator propidium iodide, and followed the effect over 13 hours. In agreement with the previous study (Haagensen *et al.*, 2007), the *pmr*-operon was not expressed in the absence of colistin (Fig. 6A t_0). The *pmr*-operon was induced after 4 hours but only in the subpopulation of cells comprising the upper layer of the biofilm, which had just been identified to exhibit high metabolic activity (Fig. 6A). No induction was observed in the interior part of the biofilm. Instead, cells in this part, which were not

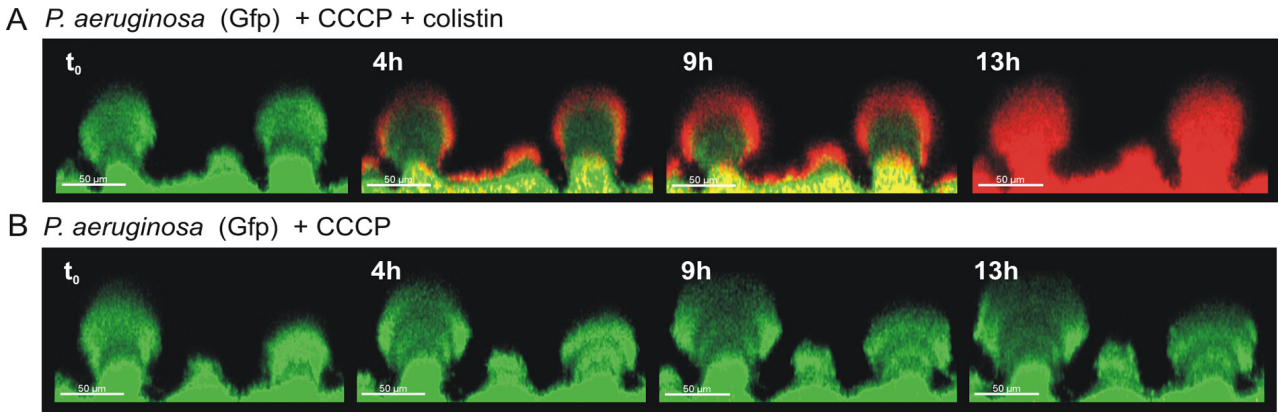

Fig. 5. Biofilm cells exposed to CCCP become sensitive to colistin. Biofilms of *P. aeruginosa* PAO1 Gfp were grown for 4 days and then continuously exposed to either 25 µg/ml colistin, 30 µM CCCP and propidium iodide (A), or, as control, to 30 µg/ml CCCP and propidium iodide (B). Confocal laser scanning micrographs were acquired at time point t_0 (prior to exposure) and 4, 9, and 13 hours subsequent to the beginning of treatment. The images represent vertical sections of biofilms, respectively. Live cells appear green due to expression of Gfp and dead cells appear red, due to staining with the dead-cell indicator propidium iodide.

able to induce the *pmr*-operon during the first hours upon colistin exposure, were killed by the colistin subsequently (Fig. 6A). To verify that all cells in the stalk-part were killed by colistin, we exposed the biofilm to Syto 9, a fluorescent indicator-dye visualizing live cells. However, no living cells were detected in the stalk part of the biofilm (data not shown), which is in agreement with the similar experiment presented in Fig. 1B. According to the results described in the previous sections, biofilm cells depleted of metabolic energy should not be able to induce the *pmr*-operon. And indeed, when a 4-day-grown biofilm of *P. aeruginosa* P*pmrH-gfp* was exposed simultaneously to 30 µg/ml CCCP and 25 µg/ml colistin in the presence of propidium iodide, no induction of the *pmr*-operon was observed

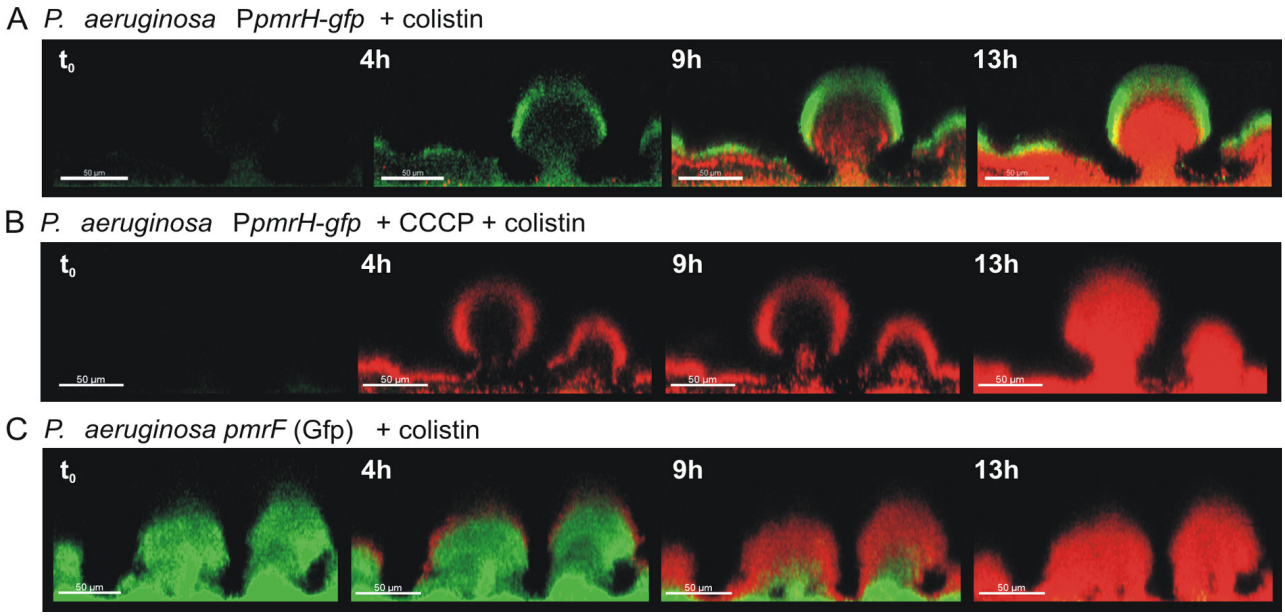

Fig. 6. Expression of the *pmr*-operon and cell-death upon colistin exposure in *P. aeruginosa* biofilms takes place in a spatiotemporal-dependent manner. Biofilms of *P. aeruginosa* PAO1 Tn7-P*pmrH-gfp* (A + B) and *P. aeruginosa* PAO1 *pmrF* (Gfp) (C) were grown for 4 days and then continuously exposed to either 25 µg/ml colistin and propidium iodide (A + C), or to 25 µg/ml colistin, 30 µM CCCP and propidium iodide (B). Confocal laser scanning micrographs were acquired at time point t_0 (prior to exposure) and 4, 9, and 13 hours subsequent to the beginning of treatment. The images represent vertical sections of biofilms, respectively. Live cells appear green due to inducing expression of the *pmr*-operon (A + B) or constitutively expression of Gfp (C) and dead cells appear red, due to staining with the dead-cell indicator propidium iodide.

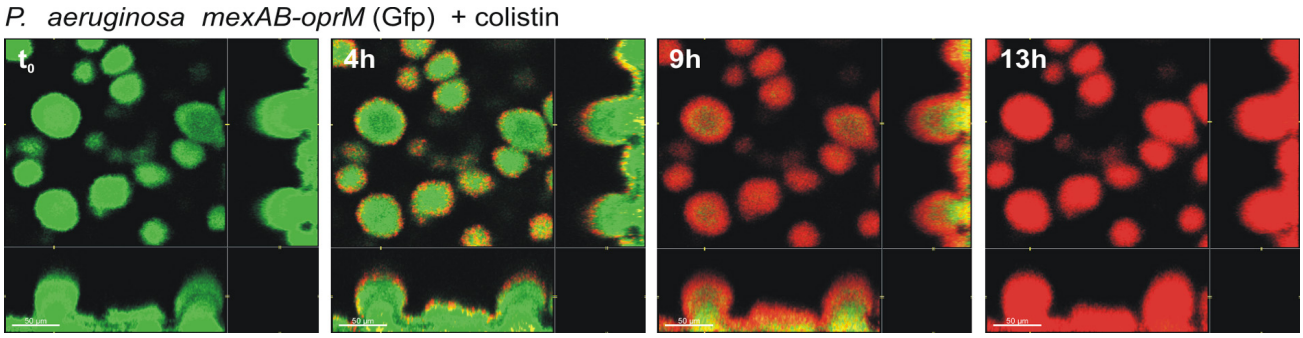

Fig. 7. *P. aeruginosa* cells devoid of MexAB-OprM exhibit increased sensitivity to colistin in biofilms. Biofilms of *P. aeruginosa* PAO1 Δ*mexAB-oprM*::Gm^r Gfp were grown for 4 days and then continuously exposed to 25 μg/ml colistin and propidium iodide. Confocal laser scanning micrographs were acquired at time point t_0 (prior to exposure) and after 4, 9, and 13 hours subsequent to treatment. The images show a horizontal section with two flanking images representing sections in the xz and yz planes, respectively. Live cells appear green due to expression of Gfp and dead cells appear red, due to staining with the dead-cell indicator propidium iodide.

(Fig. 6B). Consequently, the cells in the upper layer of the biofilm, as well as in the interior part were killed (Fig. 6B). In agreement with the previous report by Haagensen *et al.* (2007), also a mutant, which has a defect in the *pmr*-operon (such as a *pmrF*-mutant), was not able to develop tolerance towards colistin in biofilms, and therefore almost all cells were killed within 13 hours (Fig. 6C).

mexAB-oprM is required for tolerance towards colistin

The uncoupler of oxidative phosphorylation CCCP was used in the experiments described above to interfere with energy-driven cellular metabolic/ physiological processes by disrupting the proton motive force (PMF). However, a disruption of PMF in bacterial cells does not only inhibit the regeneration of redox equivalents, it also interferes with the energy-driven transport of small molecules across the membrane such as the transport mediated by some efflux pumps. CCCP is therefore also frequently used as efflux pump inhibitor to study the role of efflux pumps in resistance to antimicrobial compounds (e.g. Takiff *et al*., 1996; Bogdanovich, *et al*., 2006). *P. aeruginosa* is known for its intrinsic resistance to antimicrobials, to a large degree mediated by efflux pumps (Schweizer, H.P., 2003; Poole, K., 2005). However, although so far no efflux pump has been described to confer tolerance towards colistin in *P. aeruginosa*, we wanted to assess this. Consequently, we constructed a number of RND-efflux-pump knock-out mutants, namely *mexPQ-oprE* (PA3521-3523), *mexAB-oprM* (PA0425-0427), *mexGHI-oprD* (PA4205-4208), and *yegMNO-opmB* (PA2525-2528), and tested their ability to develop tolerance to colistin when grown as biofilms. We found that specifically the *mexAB-oprM* mutant exhibited a significant decrease in tolerance to colistin in biofilms. When a 4-day-grown biofilm of the *P. aeruginosa mexAB-oprM* mutant was exposed to 25 μg/ml colistin, it was unable to develop tolerance towards colistin (Fig 7). Already after 4 hours of exposure to colistin, some cells within the upper layer of the biofilm were killed, and within 13 hours of exposure almost all cells of the biofilm were eradicated (Fig. 7). To verify this finding we tested the independently created *mexAB-oprM*-mutant *P. aeruginosa* PAO200, which has been used in several laboratories, and found that it also exhibited a significant decrease in tolerance towards colistin in biofilms compared to its isogenic wild type strain (data not shown). This indicates that the MexAB-OprM efflux pump is involved in tolerance development to colistin in *P. aeruginosa* biofilms.

Expression of mexAB-oprM is increased in the upper layer of a biofilm upon colistin exposure

To assess, whether *mexAB-oprM* is constitutively expressed in our biofilm, or induced upon colistin exposure, we introduced the fluorescent reporter fusion p*mexA-gfp* into the *P. aeruginosa* wild type strain and followed expression of the reporter in biofilms formed by this strain. A 4-day-grown biofilm of *P. aeruginosa* p*mexA-gfp* exhibited a homogenous low fluorescent signal, indicating a weak basal expression of *mexAB-oprM* in the biofilm (Fig. 8). However, upon exposure to 25 μg/ml colistin, the fluorescent signal of the cells

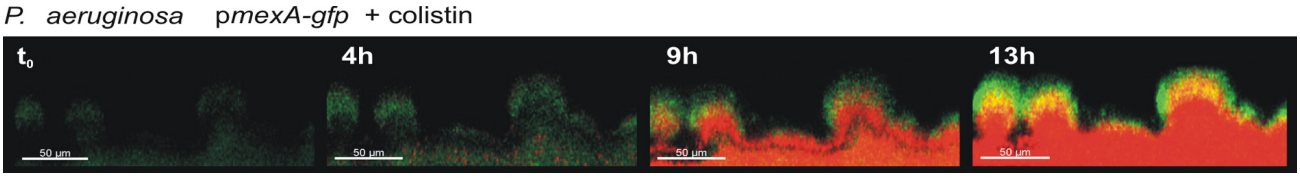

Fig. 8. Expression of *mexA* in *P. aeruginosa* biofilms. Biofilms of *P. aeruginosa* PAO1 p*mexA-gfp* were grown for 4 days and then continuously exposed to 25 µg/ml colistin and propidium iodide. Confocal laser scanning micrographs were acquired at time point t_0 (prior to exposure) and after 4, 9, and 13 hours subsequent to treatment. The images represent vertical sections of biofilms, respectively. Live cells which express *mexAB-oprM* appear green due to expression of Gfp under control of the *mexA*-promotor and dead cells appear red, due to staining with the dead-cell indicator propidium iodide.

situated in the upper layer of the biofilm increased over time (Fig. 8). Cells in the interior part of the biofilm did not exhibit an increased expression of *mexAB-oprM* and were killed within 13 hours upon exposure to colistin (Fig. 8). Biofilms of the wild type strain, harbouring the same vector, but expressing *gfp* from the *mexC*-promotor (regulating expression of the efflux pump MexCD-OprJ) instead, exhibited a lower basal expression compared to *P. aeruginosa* p*mexA-gfp,* and no induction of *mexC* upon colistin exposure (data not shown). Altogether, this indicates that colistin is a signal for up-regulation of *mexAB-oprM* expression, and that MexAB-OprM contributes to tolerance development towards colistin in *P. aeruginosa* biofilms.

Specifically targeting distinct physiological subpopulations in biofilms enables eradication of most biofilm cells using combined antimicrobial treatment

The observations described so far indicate that the biofilm is composed of at least two distinct physiological subpopulations. The subpopulation of cells situated close to the substratum exhibited low metabolic activity and sensitivity to the antimicrobial peptide colistin. The subpopulation of cells comprising the upper layer within the biofilm exhibited high metabolic activity and sensitivity to ciprofloxacin, which preferably targets replicating cells. It was therefore of interest to investigate, if a combined treatment using the two compounds ciprofloxacin and colistin, would eradicate all cells in the biofilm. To examine this, 4-day-grown biofilms of *P. aeruginosa* were established and exposed to colistin or ciprofloxacin alone, or simultaneously to ciprofloxacin and colistin for 24 hours (in the presence of propidium iodide respectively). As shown in fig. 9, a combined treatment with ciprofloxacin and colistin was able to kill almost all cells in the biofilm: Less than 10^{+1} cells/ml survived the combined antimicrobial treatment, compared to $3.80*10^{+5}$ cells/ml and $2.25*10^{+7}$ cells/ml on average in separately colistin- or ciprofloxacin-treated biofilms (Fig. 9) (details regarding the determination of the number of surviving biofilm cells are described in the Experimental procedures section). To get additional evidence, that it is possible to specifically target the two different subpopulations dependent on their metabolic activity, we chose to treat biofilms with another antimicrobial agent, which is supposed to act specifically on active cells. We exposed 4-day-grown biofilms to tetracycline, a compound interfering with bacterial translation, for 24 hours (in the presence of propidium iodide). As expected, tetracycline alone only killed the subpopulation of cells in the upper layer of the biofilm, leaving $1.90*10^{+8}$ cells/ml on average surviving (Fig. 9). However, a combined treatment with tetracycline and colistin was able to eradicate most cells in the biofilm, with a surviving fraction of $9.60*10^{+2}$ cells/ml (Fig. 9). This indicates, that by combined antimicrobial treatment, using compounds that target separate physiological subpopulations within biofilms, it is possible to kill the majority of the cells in a biofilm.

Discussion

Bacteria grown as biofilms are frequently reported to exhibit inherent tolerance to antimicrobial compounds, and might therefore contribute to the persistence of some human infections (Costerton, *et al*., 1999, Hall-Stoodley *et al*., 2004). Antimicrobial peptides can be produced by humans as part of the innate immune defence system and are increasingly administered as treatment against microbial infections in humans (Falagas and Kasiakou, 2005; Hancock, 2001; Hancock and Sahl, 2006; Li *et al*., 2006; Zasloff, 2002). So far, however, little is known about potential mechanisms, which might

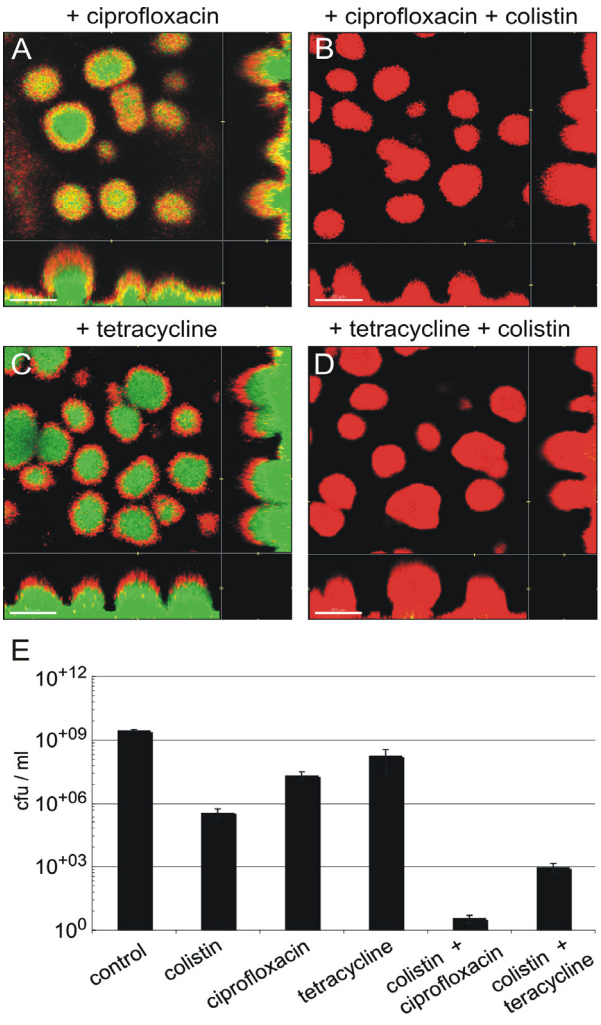

Fig. 9. Targeting distinct subpopulations in *P. aeruginosa* biofilms by single and combined antimicrobial treatment and quantification of the surviving fraction. Biofilms of *P. aeruginosa* PAO1 Gfp were grown for 4 days and then continuously exposed to either 60 μg/ml ciprofloxacin and propidium iodide (A), 60 μg/ml ciprofloxacin, 25 μg/ml colistin and propidium iodide (B), 200 μg/ml tetracycline and propidium iodide (C), or to 200 μg/ml tetracycline, 25 μg/ml colistin and propidium iodide (D) for 24 hours. The confocal laser scanning micrographs show a horizontal section with two flanking images representing sections in the xz and yz planes, respectively. Live cells appear green due to expression of Gfp and dead cells appear red, due to staining with the dead-cell indicator propidium iodide. The number of cells (cfu), which survived the antimicrobial treatment, was determined by plating and counting of cells harvested from biofilms (E). The standard deviation for each point was calculated with $n = 4$.

contribute to resistance or tolerance development in biofilms towards antimicrobial peptides.

Haagensen and colleagues recently reported that colistin kills preferably a distinct subpopulation of cells situated close to the substratum in *P. aeruginosa* PAO1 biofilms, whereas a distinct subpopulation situated on top, exhibits phenotypic tolerance to colistin (Haagensen *et al.*, 2007). In addition, our results here suggest that this might be a general feature of *P. aeruginosa* flow-chamber-grown biofilms, since in addition to the laboratory strain PAO1 also clinical isolates exhibited this phenotype under the same conditions.

Haagensen *et al.* (2007) previously presented results on 2-day-grown *P. aeruginosa* PAO1 biofilms, which strongly suggested, that cellular migration is involved in tolerance development to colistin in *P. aeruginosa* PAO1 biofilms. Surprisingly, we found in the present study that mature biofilms of various mutant strains, impaired in cellular migration, exhibited a similar tolerance phenotype as the wild type, independent of the actual three-dimensional structure of the biofilm: i.e. a colistin-sensitive subpopulation close to the substratum and a colistin-tolerant subpopulation on top. In addition, it should be noted, that also the clinical isolates used here are to different degrees impaired in cellular migration (data not shown). Altogether, this indicates, that cellular migration *per se* is not involved in tolerance development in mature *P. aeruginosa* biofilms. It might be speculated that cellular migration could be a major factor required for developmental processes during the first 2 days of biofilm formation of *P. aeruginosa* PAO1 and might be involved in colistin tolerance development (Haagensen *et al.*, 2007; Klausen *et al.*, 2003b). However, cellular migration might slow down during the following stages and structural maturing processes of the cap-forming subpopulation might then be mainly due to cell proliferation. Because of technical limitations of CLSM, it has not been possible to assess whether the cells of the cap-forming subpopulation in mature biofilms are still migrating (data not shown). More detailed investigations will be required to understand the impact of cellular migration on colistin tolerance development in young *P. aeruginosa* PAO1 biofilms.

Our results here provide evidence, that metabolic/physiological activity in the biofilm is highest in a distinct subpopulation of cells in the upper layer of the multicellular structures. This conclusion is based on experiments involving *in situ* gene expression studies using a fluorescent reporter fusion, expressing an unstable version of Gfp under the control of a ribosomal promotor. Moreover, this subpopulation exhibited increased sensitivity to antimicrobial agents interfering with bacterial replication processes (such as ciprofloxacin) and

translation processes (such as tetracycline). In addition, the antimicrobial agent tobramycin, which interferes with bacterial translation, was recently found to target specifically cells in the upper layer of a *P. aeruginosa* biofilm (Hentzer, *et al*., 2003). Altogether, these data provide evidence that metabolic activity is highest in the upper layer of *P. aeruginosa* flow-chamber-grown biofilms, whereas metabolic activity is low in the deeper layers. This seems plausible as cells in the upper layer of the multicellular structures can obtain oxygen and nutrients from the bulk liquid, in contrast to the cells in the deeper layers where concentrations of dissolved oxygen and nutrients are likely to be low. Because similar observations have been obtained for *P. aeruginosa* biofilms grown as colony or established in capillary glass tubes (Werner *et al*., 2004), the observed spatial distribution of active and non-active cells might be a general characteristic of *P. aeruginosa* biofilms.

We observed that the distinct subpopulation of cells, which exhibits high metabolic activity, is able to survive the colistin treatment, in contrast to the subpopulation exhibiting low metabolic activity. This suggested that an energy-driven adaptation response might be required which renders the cells in the upper part tolerant to colistin. Our hypothesis was supported by the finding that biofilm cells depleted of metabolic energy (using the protonophore CCCP), were unable to adapt to colistin exposure and therefore did not survive the treatment. Haagensen *et al*. (2007) recently reported that cells in the upper layer of *P. aeruginosa* biofilms exhibited an induced expression of the *pmr*-operon, after exposure to colistin for 24 hours. Here we found using *in situ* gene expression analysis that the metabolically active cells in the upper layer of the biofilm were able to induce expression of the *pmr*-operon, which allowed them to adapt to colistin and hence survive. In contrast, the subpopulation of cells situated closer to the substratum was not able to induce expression of the *pmr*-operon, and hence did not survive. In accordance, when biofilm cells were exposed to CCCP, induction of the *pmr*-operon did not occur in the presence of colistin, and therefore the cells in the confined upper part did not survive the treatment. However, disruption of the PMF by CCCP does not only inhibit metabolic/physiological processes within cells, it also inhibits H^+- and ATP-driven efflux-pumps. Although efflux-pumps have so far not been found to confer tolerance to colistin in *P. aeruginosa*, we investigated if our CCCP-treatment might have interfered with the function of efflux pumps rendering cells sensitive to colistin. We found that two independently created *mexAB-oprM*-mutants exhibited increased sensitivity to colistin compared to the wild type. Moreover, using a fluorescent reporter fusion we found that expression of *mexAB-oprM in situ* is induced upon colistin exposure. At present it is not clear whether the effect of CCCP on colistin treated biofilms is due to depletion of metabolic energy or inhibition of efflux pumps, or both.

Various compounds (such as chloramphenicol, β-lactams, macrolides, and SDS) have been identified as substrate for the MexAB-OprM efflux pump in *P. aeruginosa*, but to our knowledge no antimicrobial peptides so far (Schweizer, 2003; Poole, 2001). Until now, efflux-pumps have been found to be involved in natural resistance to antimicrobial peptides in the Gram-negative *Neisseria gonorrhoeae* and *Neisseria meningitides* (Schafer *et al*., 1998; Tzeng *et al*., 2005) and an efflux pump/potassium antiporter system has been found to be involved in resistance to polymyxin B in *Yersinia enterocolitica* (Bengoechea, *et al.*, 2000). We found that *mexAB-oprM* is not involved in inherent resistance to colistin in *P. aeruginosa*, since the minimal inhibitory concentration (MIC) to colistin (as examined by macro-dilution method and E-test) does not significantly differ between the wild type and *mexAB-oprM* mutant under our experimental conditions (data not shown). Instead, *mexAB-oprM* is involved in development of phenotypic tolerance to colistin in *P. aeruginosa* when grown as biofilm. The observation that an efflux pump might be involved in the development of colistin tolerance could indicate that colistin also has an intracellular target in addition to interfering with the membrane of *P. aeruginosa*. In agreement, results obtained from studying the interaction of polymyxins with membranes of *P. aeruginosa* have let to the conclusion that these compounds might also have cytoplasmatic targets (Zhang *et al.*, 2000).

In some cases after prolonged colistin-exposure of biofilms formed by either the *mexAB-oprM* mutants or different *pmr* mutants we observed small randomly distributed aggregates of living cells in the biofilm (data not shown). This indicates, that a small fraction of single cells had survived the colistin treatment and were then able to initiate proliferation also in the presence of colistin. These cells did not exhibit any inherent resistance to colistin (data not shown). This might suggest that in a small fraction of *pmr*-mutant cells expression of *mexAB-oprM*

might be increased and that in a small fraction of *mexAB-oprM*-mutant cells expression of the *pmr*-operon might be increased. Together, this might indicate that in the majority of the cells neither *pmr* nor *mexAB-oprM* alone is able to completely confer tolerance to colistin in biofilms, instead both systems seem to be required simultaneously under these conditions. The fact that *mexAB-oprM* is expressed to some degree also in unexposed biofilms, might indicate that the MexAB-OprM-mediated efflux contributes to an intrinsic tolerance to colistin in biofilms. Increased expression of *mexAB-oprM* during prolonged exposure to colistin might indicate that MexAB-OprM in addition is involved in long-term survival during colistin exposure. In contrast, induction of LPS-modification, mediated by the *pmr*-operon, might be a specific adaptation response to colistin. However, more detailed investigations are required to understand the contribution of LPS-modification and efflux systems on colistin tolerance in *P. aeruginosa* biofilms.

Whereas conventional antimicrobial compounds (such as ciprofloxacin and tetracycline) specifically target metabolically active biofilm cells in the upper layer, our data indicated that colistin apparently specifically targeted the biofilm cells exhibiting low metabolic activity situated in the deeper areas. To investigate if cells, which have a low growth activity, exhibit increased sensitivity to colistin, we compared exponentially growing planktonic cells with stationary phase planktonic cells with respect to sensitivity to colistin. However, the survival rate upon colistin treatment was similar under both conditions (data not shown). In a number of other batch culture experiments we addressed the potential role of oxygen- or energy-depletion or pH on cellular sensitivity to colistin, however, also under these conditions no significant difference was observed (data not shown). We did not examine conditions under which the expression of the *pmr*-operon is induced (e.g. Mg^{2+}-limitation), since we did not observe any induction of *pmr*-genes in our biofilms in the absence of colistin. Moreover, we could not observe any significant differences in sensitivity to colistin in our batch culture experiments under non-inducing conditions, when we compared wild type and a *pmrF*-mutant (data not shown), which is in agreement with previous reports (McPhee *et al*., 2003). Altogether this supports the notion, that sensitivity to polymyxins is greatly dependent on the prevailing environmental conditions. With respect to our investigations on biofilms, this might indicate on the one hand, that a complex microenvironment of unknown composition in the deeper layers of the biofilm exist, which impacts on cellular metabolic activity and sensitivity to colistin, but might be difficult to simulate in batch cultures. On the other hand, the particular subpopulation of cells exhibiting low metabolic activity in the biofilms, might at the same time exhibit another specific characteristic, independent of metabolic activity, which renders it sensitive to colistin. Interestingly, the chelator EDTA targets the same subpopulation of cells in *P. aeruginosa* biofilms as colistin (Banin *et al*., 2006; data not shown), and investigations on batch culture grown cells have indicated, that EDTA preferably targets stationary phase cells compared to exponential phase cells (Imamura *et al*., 2005). Investigations are currently ongoing in our laboratory, to determine the factors, which render cells in the deeper layers of biofilms sensitive to colistin.

Our observation that conventional antimicrobial agents (e.g. ciprofloxacin and tetracycline) specifically target the distinct subpopulation of biofilm cells in the upper layer, whereas colistin preferably targets the distinct subpopulation of biofilm cells in the deeper layers, prompted us to study the killing-effect of a combined treatment with these antimicrobial compounds. The combined treatment with either colistin + ciprofloxacin or colistin + tetracycline reduced the number of biofilm cells significantly, compared to the single anti-microbial treatments. In particular the combined treatment with colistin + ciprofloxacin was very effective, eradicating nearly all cells of the biofilm. Intriguingly, the administration of aerosolized colistin in combination with oral ciprofloxacin has been found to significantly reduce the onset of chronic *P. aeruginosa* infection in cystic fibrosis patients (Valerius *et al*., 1991). Furthermore, this treatment strategy is part of a recommended early intervention and prevention therapy of lung disease in cystic fibrosis according to a European consensus report (Döring *et al*., 2004; Høiby *et al*., 2005).

Altogether, our data indicate that in general antimicrobial tolerant cells in biofilms are not randomly distributed. Instead, antimicrobial tolerance seems to be confined to physiologically distinct subpopulations of cells within the multicellular structures, independent of the antimicrobial compound used. Tolerance development to colistin is confined to a distinct subpopulation of metabolically active cells, which is able to adapt to colistin exposure by i) reducing interaction with the

antimicrobial compound (LPS-modification mediated by the *pmr*-operon), and ii) export of the antimicrobial compound (efflux mediated by *mexAB-oprM*). Moreover, we found that a systematic combined antimicrobial treatment, specifically targeting distinct physiological subpopulations, enables eradication of almost all cells in a biofilm.

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Appendix

Paper 4

Barken, K. B., **Pamp, S. J.**, Yang, L., Klausen, M., Givskov, M., Bertrand, J., Whitchurch, C., Engel, J., and Tolker-Nielsen, T. Roles of Type IV Pili, Flagella, and Extracellular DNA in Structural Development of *Pseudomonas aeruginosa* Biofilms.

This manuscript is in preparation. It is set up as a two column layout for lucidity purposes.

Roles of type IV pili, flagella, and extracellular DNA in *Pseudomonas aeruginosa* biofilm development.

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Detailed knowledge of developmental processes that leads to the formation of complex multicellular structures in biofilms may be useful for creating strategies to control biofilm development. We previously presented evidence that formation of the cap-portion of mushroom-shaped structures in *Pseudomonas aeruginosa* biofilms occurs via type IV pili-driven bacterial motility (Mol. Microbiol. 50:61-68). In the present study we present evidence that, although type IV pili are required for the formation of mushroom caps in *P. aeruginosa* biofilms, flagellum-driven motility is responsible for accumulation of the bacteria in the mushroom caps. In addition, we present evidence that extracellular DNA and quorum-sensing play a role in mushroom cap formation. The presented data are consistent with a model suggesting that formation of the mushroom shaped structures in *P. aeruginosa* biofilms occurs in a developmental process involving formation of initial microcolonies by a non-motile subpopulation that releases extracellular DNA, and subsequently become capped by a motile subpopulation that uses flagella for surface associated motility and type IV pili for binding to the extracellular DNA.

Introduction

During the last ten years there has been a rapidly increasing recognition of microbial biofilms as a highly significant topic in microbiology with relevance for a variety of areas in our society including the environment, industry, and human health. Growing appreciation of the importance of biofilms has led to the perception that these communities constitute the dominant mode of microbial life (Hall-Stoodley *et al*., 2004; Davey and O'Toole, 2000). Evidence is increasing that biofilms are complex and dynamic communities in which substantial phenotypic diversification allows microorganisms to adapt to different environments (Watnick and Kolter, 2000; Klausen *et al*., 2006). Understanding the pathways to biofilm development will eventually make it possible to manipulate the growth of biofilms in nature and disease.

The use of flow-chamber in vitro setups and confocal laser scanning microscopy (CLSM) has provided knowledge about mechanisms involved in biofilm formation. *Pseudomonas aeruginosa*, a model organism in biofilm research, can, dependent on the conditions, form biofilms with different spatial structures. For example, it has been reported that a flat biofilm was formed in flow-chambers irrigated with citrate minimal medium (Klausen *et al.*, 2003b), while a heterogeneous biofilm with mushroom-shaped multicellular structures was formed in flow-chambers irrigated with glucose minimal medium (Klausen *et al.*, 2003a). *P. aeruginosa* biofilm development in flow-chambers with citrate as carbon source was shown to occur via formation of initial microcolonies by clonal growth of sessile cells at the substratum, followed by expansive migration of the bacteria on the substratum, and the subsequent formation of a flat biofilm (Klausen *et al.*, 2003b). Because biofilm formation by a *P. aeruginosa pilA* mutant (deficient in biogenesis of type IV pili) occurred without the expansive phase, and resulted in discrete protruding microcolonies, it was suggested that the expansive migration of the bacteria was type IV pili-driven. Moreover, it was shown that a *P. aeruginosa fliM* mutant (deficient in assembly of flagella) formed a hilly biofilm in flow-chambers with citrate as carbon source, indicating that flagella may play a role in surface-associated migration in *P. aeruginosa* biofilm in addition to type IV pili (Klausen *et al.*, 2003b). Evidence has been provided that the

formation of mushroom-shaped structures in glucose-grown *P. aeruginosa* biofilms occurs in a sequential process involving a non-motile bacterial subpopulation which forms the initial microcolonies by growth in certain foci of the biofilm, and a migrating bacterial subpopulation which initially forms a monolayer on the substratum, and subsequently forms the mushroom caps by aggregating on top of the initial microcolonies (which then become mushroom stalks) via a type IV pili-dependent process (Klausen *et al.*, 2003a). In glucose-grown biofilms, that was initiated with a mixture of Cfp-tagged and Yfp-tagged *P. aeruginosa* wild-type bacteria, mushroom-shaped structures were formed that had single-colour stalks indicating their formation via clonal growth, and two-colour caps indicating their formation via aggregation of bacteria. In *pilA*/wild-type mixed biofilms formation of mushroom-shaped structures occurred both with citrate and glucose as carbon source. In citrate-grown *pilA*/wild-type mixed biofilms the *pilA* bacteria formed stalks, and the wild-type bacteria accumulated on top of these stalks and formed caps. In glucose-grown *pilA*/wild-type mixed biofilms either the *pilA* bacteria (most frequent) or the wild-type bacteria formed the stalks, and the wild-type bacteria accumulated on top of these stalks and formed the caps. Subsequently experiments involving microscopic tracking of fluorescent and non-fluorescent bacteria provided direct evidence that glucose-grown *P. aeruginosa* wild-type biofilms contain a non-motile stalk-forming subpopulation and a motile cap-forming subpopulation (Haagensen *et al*., 2007).

The extracellular matrix that holds the cells together in *P. aeruginosa* biofilms mainly consists of polysaccharide, protein, and DNA (Pamp *et al*., 2007). Evidence has been presented that a basal level of extracellular DNA in *P. aeruginosa* populations is generated via a pathway which is not linked to quorum-sensing, while a larger amount of extracellular DNA appears to be generated via a pathway that depends on quorum-sensing and results in lysis of a small subpopulation of the cells (Allesen-Holm *et al.*, 2006). The *P. aeruginosa* quorum-sensing mutants *lasIrhlI* and *pqsA* formed biofilms with low extracellular DNA levels and increased susceptibility to treatment with SDS, indicating a stabilizing effect of the extracellular DNA in the biofilms (Allesen-Holm *et al.*, 2006). In addition, mature wild-type biofilms that had been treated with DNase for a short time showed elevated susceptibility to the SDS treatment (Allesen-Holm *et al.*, 2006). The extracellular DNA in *P. aeruginosa* biofilms appears to be organized in distinct patterns (Allesen-Holm *et al*., 2006). In young glucose-grown *P. aeruginosa* biofilms the extracellular DNA was shown to be present in high concentrations specifically in the outer layer of the microcolonies and upon the microcolonies (which subsequently become stalks). In mature glucose-grown *P. aeruginosa* biofilms the extracellular DNA was shown to be present in high concentrations specifically in the outer layer of the stalks and between the stalk and cap portion of the mushroom-shaped structures (Allesen-Holm *et al.*, 2006). In agreement with the spatial distribution of the extracellular DNA and a role of quorum-sensing in DNA release, experiments involving a *P. aeruginosa pqsA-gfp* reporter strain suggested that the *pqs* genes were expressed specifically in the outer layer of the stalks that subsequently became capped by the migrating subpopulation (Yang *et al*., 2007).

Based on agar plate assays two kinds of surface associated motility have been defined for *P. aeruginosa*. One kind of surface-associated motility which requires type IV pili has been termed twitching motility (Henrichsen, 1972; Semmler, *et al*., 1999; Mattick, 2002). Another kind of surface-associated motility which requires functional flagella, biosurfactant production, and under some conditions type IV pili, has been termed swarming motility (Köhler *et al*., 2000; Rashid and Kornberg, 2000; Déziel *et al*., 2003). Comparative sequence analysis has suggested that *P. aeruginosa* encodes four chemotaxis-like signal transduction systems (Stover *et al*., 2000). The Pil-Chp system (PA0408-PA0417) is evidently involved in regulating twitching motility (Darzins, 1994; Whitchurch *et al*., 2004). The Che (PA1464-PA1456 and PA3348-PA3349) and Che2 (PA0173-PA0179) systems, both homologous to the *E. coli* Che chemotaxis system (Parales *et al*., 2004), have been implicated in flagella-mediated chemotaxis in *P. aeruginosa* (Kato *et al*., 1999; Ferrandez *et al*., 2002; Hong *et al*., 2004; 2005). The Wsp system (PA3702-3708) appears to control the expression of Cup fimbria (D'Argenio *et al*., 2002), and the PEL and PSL polysaccharides (Hickman *et al*., 2005), which are all compounds that have been implicated in *P. aeruginosa* biofilm formation (Vallet *et al*., 2001; Jackson *et al*., 2004; Matsukawa and Greenberg, 2004; Friedman and Kolter, 2004).

The cap-forming and the stalk-forming sub-population of the mushroom-shaped structures in *P.*

aeruginosa biofilms in many cases display differential tolerance to antimicrobial compounds. For example, the antibiotics tobramycin, ciprofloxacin, and tetracycline were shown to kill preferentially bacteria located in the cap-portion of the mushroom-shaped structures, whereas the antibiotic colistin, the detergent SDS, and the chelator EDTA were shown to kill preferentially bacteria in the stalk-portion of the mushroom-shaped structures (Bjarnsholt *et al.*, 2005; Banin *et al.*, 2006; Haagensen *et al.*, 2007; Pamp *et al.*, submitted). Evidence has been presented that *P. aeruginosa* biofilms that do not develop normally, either because the bacteria are mutated or because specific compounds are present, do not show the characteristic tolerance patterns after treatment with various antimicrobials including tobramycin, ciprofloxacin, colistin, and EDTA (Bjarnsholt *et al.*, 2005; Haagensen *et al.*, 2007; Yang *et al.*, 2007). Because the structure of a biofilm evidently can influence its antimicrobial tolerance properties, knowledge about structural biofilm development may be useful for creating strategies to control biofilm formation. The present study is focussed on elucidating factors which are involved in the formation of the mushroom-shaped structures in *P. aeruginosa* biofilms.

Materials and Methods

Bacterial strains and growth conditions

P. aeruginosa PAO1 (Holloway and Morgan, 1986) from John Mattick's laboratory was used as the wild type strain in the present study. The *pilA*, *fliM* and *pilAfliM* derivatives were constructed by allelic displacement as described by Klausen *et al.* (2003b). The *pilAlasRrhlR* triple mutant was derived from the *pilA* mutant via allelic exchange as described by Beatson *et al.* (2002). The *chpA* in frame deletion mutant was constructed by allelic displacement as described by Whitchurch *et al.* (2004). The *chpB* mutant was constructed as described below. The strains were fluorescently tagged at an intergenic neutral chromosomal locus with *gfp*, *cfp* or *yfp* in mini-Tn*7* constructs as described by Klausen *et al.* (2003b). Modified FAB medium (Heydorn *et al.*, 2000) was used supplemented with 30 mM glucose for batch overnight cultures, and with 0.3 mM glucose for biofilm cultivation. Biofilms and batch cultures were grown at 30^{o}C.

Construction of the P. aeruginosa chpB in frame deletion mutant

The *chpB* mutant was constructed in two steps. First, the gene was replaced by a gentamycin resistance (Gen^{R}) cassette. Second, the cassette was crossed out using an untagged deletion construct to create the unmarked in frame deletion. This approach was taken to facilitate the identification of mutants, which do not have an obvious twitching motility defect on agar plates.

The 5' *chpB* deletion construct was amplified from PAO1 genomic DNA using the cbpB1/chpB2.1 PCR primer pair. The 3' *chpB* deletion construct was amplified from PAO1 genomic DNA using the chpB3/chpB4 PCR primer pair. PCR products were A-tailed and ligated into pGEM-T to form pJB51 (5' deletion construct) and pJB52 (3' deletion construct). The 5' deletion construct was excised from pJB51 as an EcoRI/HindIII fragment. The 3' deletion construct was excised from pJB52 as a HindIII/PstI fragment (an overlapping Dam methylation site prevented us from using the XbaI site designed into the primer). Excised 5' and 3' deletion construct fragments were concatamerized and cloned as an EcoRI/PstI fragment into pOK12 to form pJB64. The unmarked deletion construct was excised from pJB64 as a SpeI fragment and ligated into the allelic exchange vector pJEN34 to form pJB91. A pJB95-derived Gen^{R}-cassette (Gen^{R}-cassette from pX1918GT cloned as a HindIII fragment into pOK12) was ligated as a HindIII fragment into pJB64 to create pJB73. The Gen^{R}-tagged deletion construct was excised from pJB73 as a SpeI fragment and ligated into the allelic exchange vector pJEN34 to form pJB82. pJB82 was transformed into S17.1 and transformants were mated to PAO1 as previously described to create *chpB*-Gen^{R} intermediates. pJB91 was transformed into S17.1 and transformants were mated to PAO1 *chpB*-Gen^{R} as previously described (Whitchurch *et al.*, 2004) to create unmarked Δ*chpB* mutants. This procedure deleted amino acids 37-244 of ChpB. Sequences of the primers used will be supplied upon request.

Cultivation of biofilms

Biofilms were grown in flow chambers with individual channel dimensions of 1 × 4 × 40 mm. The flow system was assembled and prepared as described previously (Sternberg and Tolker-Nielsen, 2005). The flow-chambers were inoculated by

injecting 350 µl of overnight culture diluted to an OD_{600} of 0.001 into each flow-channel with a small syringe. After inoculation flow-channels were left without flow for 1h, after which medium flow (0.2 mm s^{-1}) was started using a Watson Marlow 205S peristaltic pump. In one experiment the biofilm medium was supplemented with 100 µg/ml DNase I (Sigma) after 48 hours of biofilm cultivation.

Microscopy and image acquisition

All microscopic observations and image acquisitions were done using a Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with detectors and filter sets for monitoring of Gfp, Cfp, and Yfp fluorescence. Images were obtained using a 63x/1.4 oil objective or a 40x /1.3 oil objective. Simulated 3-D images and sections were generated using the IMARIS software package (Bitplane AG, Zürich, Switzerland).

Results

Involvement of type IV pili and the Pil/Chp chemosensory system in structural development of P. aeruginosa biofilms

Because local consumption may create nutrient gradients in biofilms (DeBeer *et al*., 1994; Picioreanu *et al*., 1998), we speculated that the motile bacteria in glucose-grown *P. aeruginosa* biofilms might accumulate on top of the initial microcolonies through the use of the type IV pili-coupled Pil/Chp chemosensory system. To test this hypothesis we constructed an in frame *P. aeruginosa chpB* deletion mutant. The *chpB* gene encodes a predicted MCP demethylase, and *chpB* mutants were previously shown to be able to move by the use of type IV pili in a Petri dish-based assay, but to be unable to display normal regulation of this motility, presumably due to a defect in relaxation of the chemosensory system (Whitchurch *et al*., 2004). We performed investigations of biofilm development of the *P. aeruginosa chpB* mutant in flow-chambers irrigated with glucose-minimal medium. During biofilm development the *chpB* mutant was able to differentiate into a non-motile subpopulation and a motile subpopulation. The non-motile subpopulation formed micro-colonies, however the motile subpopulation did not form caps such as the wild-type but rather covered the microcolonies (Fig. 1A-D). Similarly, when the

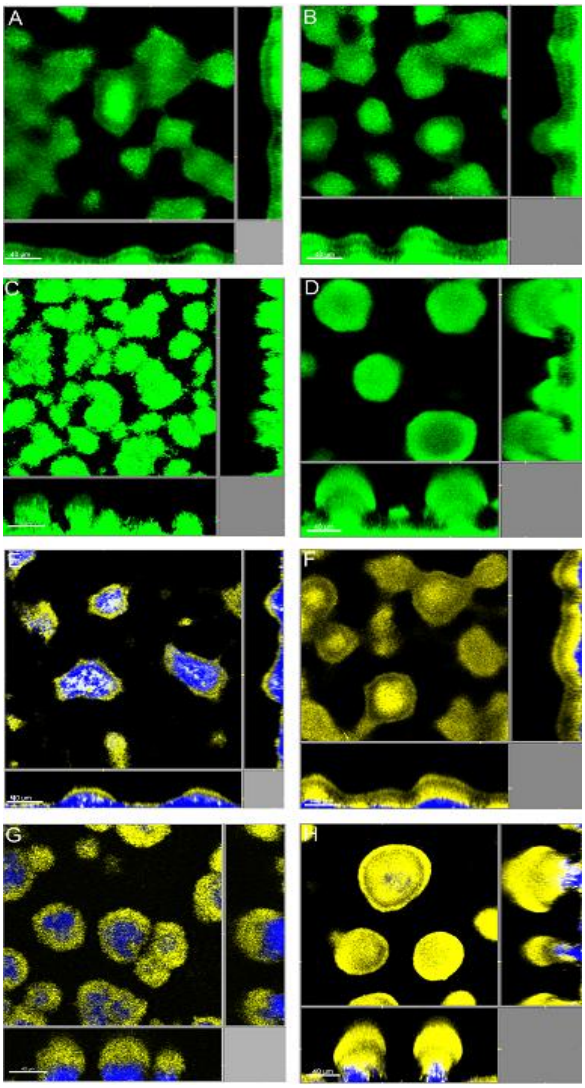

Fig. 1. Confocal laser scanning micrographs of 2-day-old (A, C, E, G) and 4-day-old (B, D, F, H) biofilms formed by *P. aeruginosa chpB* Gfp (A, B), *P. aeruginosa* wild type Gfp (C, D), a mixture of *P. aeruginosa chpB* Yfp and *P. aeruginosa pilA* Cfp (E, F), and a mixture of *P. aeruginosa* wild type Yfp and *P. aeruginosa pilA* Cfp (G, H). The central images show horizontal optical sections and the flanking images show vertical optical sections. The bars represent 40 µm.

chpB mutant was cultivated in mixed biofilms together with a *pilA* mutant, the *chpB* mutant did not form caps on top of the *pilA* stalks such as the wild-type, but rather covered the *pilA* microcolonies (Fig. 1E-H).

During the course of our investigations of the role of the Pil/Chp chemosensory system in *P. aeruginosa* biofilm development we also studied a *P. aeruginosa chpA* in frame deletion mutant. The *chpA* gene encodes a composite response regulator, and evidence has been provided that *chpA* mutants are deficient in signal sensing through the Pil/Chp chemosensory system (Whitchurch *et al*., 2004).

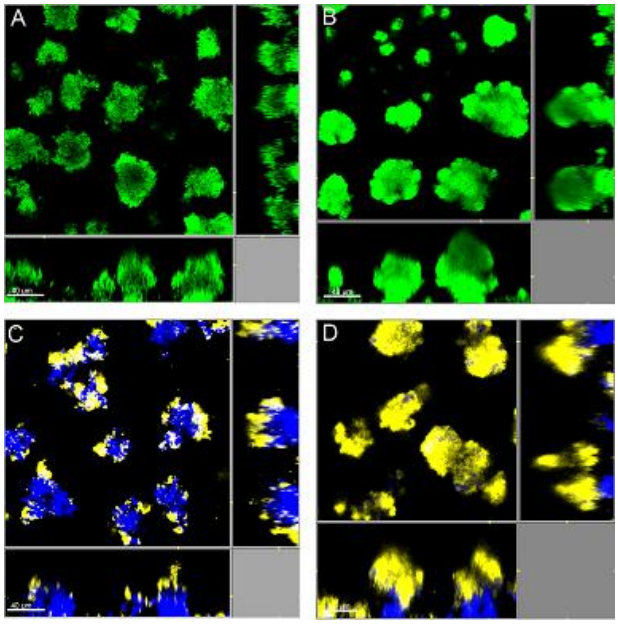

Fig. 2. Confocal laser scanning micrographs of 2-day-old (A, C) and 4-day-old (B, D) biofilms formed by *P. aeruginosa chpA* Gfp (A, B), and a mixture of *P. aeruginosa chpA* Yfp and *P. aeruginosa pilA* Cfp (C, D). The central images show horizontal optical sections and the flanking images show vertical optical sections. The bars represent 40 μm.

The PAO1 *chpA* mutant used in the present study was previously shown to be deficient in twitching motility in a Petri dish-based assay (Whitchurch *et al*., 2004). It was therefore surprising to us that this mutant was able to form (irregular) mushrooms in mono-strain biofilms (Fig. 2A, B), and (irregular) caps in *chpA*/*pilA* mixed biofilms (Fig. 2C, D).
From the experiments with the *chpB* mutant we conclude that the Chp chemosensory system plays a (minor) role in the formation of the mushroom-shaped multicellular structures in *P. aeruginosa* biofilms. The experiments with the *chpA* mutant appears to disprove our previous suggestion that cap formation in *P. aeruginosa* biofilms occurs via type IV pili-driven motility (Klausen *et al*., 2003a). However, because the *chpA* mutant was shown to be sensitive to pili-specific phages it is evidently piliated (Whitchurch *et al*., 2004). Because the *chpA* and *chpB* mutants, unlike the *pilA* mutant, evidently are piliated, our new results lead to the suggestion that cap formation in *P. aeruginosa* biofilms depends on the presence of type IV pili on the cap-forming bacteria.

Involvement of flagella in structural development of P. aeruginosa biofilms

Because our previous investigations clearly indicated a role of surface-associated motility in mushroom cap formation in *P. aeruginosa* biofilms (Klausen *et al*., 2003a; Haagensen *et al*., 2007), and the experiments described above indicated that a *P. aeruginosa* mutant deficient in type IV pili-driven motility can form (irregularly shaped) mushroom caps, we investigated a role of flagellum-driven surface-associated motility in structural development of *P. aeruginosa* biofilms. We have previously presented evidence that in flow-chambers irrigated with citrate medium the *P. aeruginosa* wild-type displays extensive surface-associated motility and forms a flat biofilm, while the non-flagelated *P. aeruginosa fliM* mutant forms a hilly biofilm, and the non-piliated *P. aeruginosa pilA* mutant forms irregular protruding microcolonies, suggesting that surface-associated motility in citrate-grown *P. aeruginosa* PAO1 biofilms mainly depends on type IV pili, although flagella evidently also play a role (Klausen *et al*., 2003b). In order to investigate a role of flagella in the formation of the mushroom-shaped biofilm structures we studied biofilm formation of the *P. aeruginosa fliM* mutant in flow-chambers irrigated with glucose medium. As shown in Fig. 3A and 3B, the *P. aeruginosa fliM* mutant could not form mushroom-shaped structures in flow-chambers with glucose as carbon source. Furthermore, in mixed *fliM*/*pilA* biofilms the *fliM*

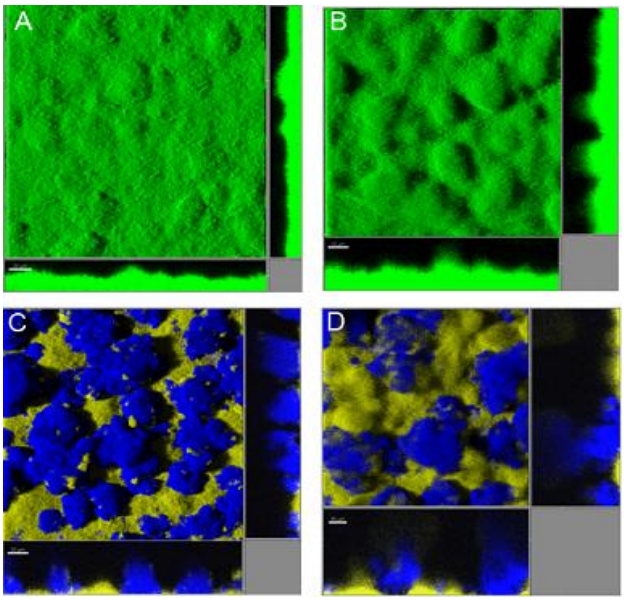

Fig. 3. Confocal laser scanning micrographs of 2-day-old (A, C) and 4-day-old (B, D) biofilms formed by *P. aeruginosa fliM* Gfp (A, B), and a mixture of *P. aeruginosa fliM* Yfp and *P. aeruginosa pilA* Cfp (C, D). The central images show top-down views and the flanking images show vertical optical sections. The bars represent 20 μm.

mutant did not form caps on top of *pilA* microcolonies (Fig. 3C, D). The wild-type/*pilA* control biofilm (not shown) developed as shown in Fig 1G and 1H. These experiments with mono-strain and mixed strain biofilms therefore suggest a role of flagellum driven motility in the formation of mushroom-shaped structures in *P. aeruginosa* biofilms.

Involvement of extracellular DNA in structural development of P. aeruginosa biofilms

The work described above indicated that formation of the cap of the mushroom-shaped structures in *P. aeruginosa* biofilms is dependent on flagellum driven motility. This finding, however, does not explain the requirement for type IV pili in cap-formation, suggested by the fact that *P. aeruginosa pilA* mutants are deficient in the process. Because type IV pili have been shown to bind with high affinity to DNA (Aas *et al.*, 2002; Van Schaik *et al.*, 2005), we found it of interest to investigate whether this component of the *P. aeruginosa* biofilm matrix might play a role in the formation of the mushroom-shaped structures. We speculated that the large amounts of extracellular DNA present on the microcolonies (stalks) in young *P. aeruginosa* biofilms (Allesen-Holm *et al.*, 2006) might cause accumulation of the migrating piliated bacteria, and thereby play a role in the formation of the mushroom caps.
Although the extracellular DNA in *P. aeruginosa* PAO1 biofilms has a stabilizing function (Whitchurch *et al*., 2002; Allesen-Holm *et al*., 2006), we have found that under the conditions used in the present study DNase treatment of 2-day-old *P. aeruginosa* PAO1 biofilms does not result in dispersal of the biofilms. We hypothesized that, if the extracellular DNA present on the microcolonies in 2-day-old *P. aeruginosa* biofilms plays a role in accumulation of the cap-forming bacteria, then DNase treatment of the biofilms from day 2 might inhibit cap formation. Fig. 4A and 4C shows CLSM micrographs acquired in a 4-day-old *P. aeruginosa* wild-type/*pilA* mixed biofilm that had been treated with DNase from day 2. It is evident that the DNase treatment reduced cap formation compared to the *P. aeruginosa* wild-type/*pilA* mixed biofilm that was not subjected to DNase treatment (Fig. 4B and 4D). In addition the DNase treatment resulted in an unusual biofilm structure where the wild-type bacteria formed bridges between the *pilA* microcolonies (Fig. 4).

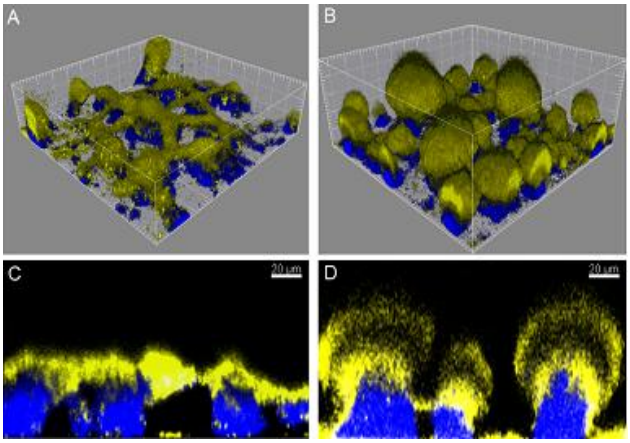

Fig. 4. Confocal laser scanning micrographs of 4-day-old biofilms formed by mixtures of *P. aeruginosa pilA* Cfp and *P. aeruginosa* wild type Yfp with (A, C) or without (B, D) DNase treatment after two days of biofilm growth. The bars represent 20 µm.

We also studied the role of extracellular DNA in the development of the mushroom-shaped *P. aeruginosa* biofilm structures via a genetic approach. We hypothesized that if a *pilA* mutant carried additional mutations so that it was unable to release extracellular DNA it would form microcolonies that could not be capped by the wild-type in mixed biofilms. We have previously presented evidence that a *P. aeruginosa lasIrhlI* quorum sensing mutant releases considerably less DNA in biofilms than the wild-type (Allesen-Holm *et al*., 2006). It was therefore of interest to investigate whether a non-piliated quorum-sensing mutant would be capped by the wild-type in mixed biofilms. We therefore constructed the non-piliated *P. aeruginosa pilA-lasRrhlR* mutant, which is deficient both in production of and response to homoserine lactones, and investigated biofilm formation of this mutant in combination with the wild type. In wild type/*pilAlasRrhlR* mixed biofilms the wild-type was unable to form normal caps on top of the *pilAlasRrhlR* microcolonies (Fig. 5). The mushroom-shaped structures that were observed contained wild-type in both the stalk and the cap (Fig. 5). The wild-type/*pilA* control biofilm (not shown) developed as shown in Fig. 1H and 4B. In order to obtain an objective measure of the role of quorum-sensing and DNA release in the development of mushroom-shaped structures, we acquired a large number of CLSM images at random positions in 4-day-old biofilms of wild-type/*pilAlasRrhlR* and wild-type/*pilA*, and assessed whether the *pilA-lasRrhlR* and *pilA* microcolonies were colonized by the wild-type bacteria. In the wild-type/*pilAlasRrhlR*

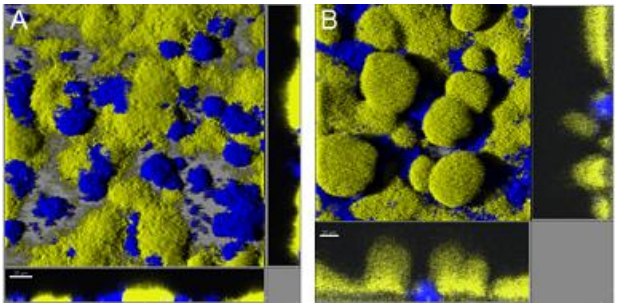

Fig. 5. Confocal laser scanning micrographs of a 2-day-old (A) and 4-day-old (B) biofilm formed by a mixture of *P. aeruginosa pilAlasRrhlR* Cfp and *P. aeruginosa* wild type Yfp. The central images show top-down views and the flanking images show vertical optical sections. The bars represent 20 μm.

biofilm 6 out of 200 *pilAlasRrhlR* microcolonies were colonized by the wild-type, whereas in the wild-type/*pilA* biofilm 198 out of 200 *pilA* microcolonies were capped by the wild-type.
Together these experiments suggest that quorum-sensing and DNA-release in the initial micro-colonies in *P. aeruginosa* biofilms plays a role in the subsequent development of mushroom-shaped structures.

Discussion

We previously suggested that formation of the cap-portion of the mushroom-shaped structures in *P. aeruginosa* biofilms occurs via type IV pili-driven motility (Klausen *et al.*, 2003b). This suggestion was based on evidence that cap-formation occurs through aggregation of bacteria and requires type IV pili. The present study has shown that our previous model for the development of the mushroom-shaped structures in *P. aeruginosa* biofilms needs revision. The major findings that form the basis for a new model indicate that: i) non-motile bacteria form the stalks (initial microcolonies) and motile bacteria subsequently form the caps (Klausen *et al*., 2003b; Haagensen *et al*., 2007), ii) The bacteria in the outer layer of the stalks release extracellular DNA (Allesen-Holm *et al*., 2006; Yang *et al*., 2007). iii) cap formation depends on type IV pili (Klausen *et al*., 2003b), iv) piliated non-twitching bacteria can form caps (this study), v) cap formation depends on flagellum-driven motility (this study), and vi) cap formation depends on extracellular DNA released by the stalk-subpopulation (this study). In accord with this evidence we suggest the following model for the formation of the mushroom-shaped structures in *P. aeruginosa* biofilms: i) Non-motile bacteria form the mushroom stalks by growth in certain foci of the biofilm. ii) The bacteria in the outer layer of the stalks release extracellular DNA. iii) Migrating bacteria climb the stalks in a process which is driven by flagella. iv) The migrating bacteria bind to the extracellular DNA on the stalks via type IV pili and form caps.

Stalk formation may initiate in certain foci of a *P. aeruginosa* biofilm as a consequence of down-regulation of motility in a subpopulation of the cells. In agreement with a requirement of down-regulation of twitching motility for initial microcolony formation, it has been reported that lactoferrin inhibits the formation of microcolonies in *P. aeruginosa* biofilms by preventing down-regulation of twitching motility (Singh *et al*., 2002). In addition, evidence has been presented that microcolony formation in *P. aeruginosa* biofilms can be prevented by carbon-source dependent stimulation of type IV pili-driven motility (Klausen *et al.*, 2003a) or flagella-driven motility (Shrout *et al*., 2006). However, it is also possible that microcolony formation initiates because the cells in certain foci of the biofilms produce matrix components and adhere strongly to each other so that motility becomes arrested.

In agreement with our suggestion that type IV piliation of the cells is required for mushroom cap formation, whereas type IV pili-driven motility is not necessary, Chiang and Burrows (2003) reported that a *P. aeruginosa pilT* mutant, which is hyper-piliated and twitching deficient, formed biofilms with large mushroom-shaped structures.

The finding that the *P. aeruginosa chpB* mutant did not form normal caps, but rather covered the stalks, and the finding that the *P. aeruginosa chpA* mutant formed caps of irregular shape, could suggest that type IV pili-driven motility plays a role in cap formation a long with flagella-driven motility. However, Caiazza *et al*. (2007) recently provided evidence that *P. aeruginosa chpB* mutants display reduced flagella-driven swarming motility, which may alternatively explain the finding that the *chpB* mutant was found to be affected in cap-formation in the present study. In addition, evidence has been provided that the *chpA* mutant used in the present study display reduced surface piliation (Whitchurch *et al*., 2004), which might affect the suggested interaction with extracellular DNA and thereby cap formation.

The involvement of quorum sensing in *P. aeruginosa* biofilm development has been studied by crudely monitoring biofilm structures, and appears to play a role only under some conditions

(Davies *et al*., 1998; Stoodley *et al*., 1999; Purevdorj *et al*. 2002; Heydorn *et al*., 2002). Obviously the outcome of an analysis is restricted by its level of refinement, and quorum sensing could play roles in a biofilm that apparently does not affect its structure. In the present investigation the use of a model system consisting of Cfp-tagged stalk-formers and Yfp-tagged cap-formers provided evidence that quorum-sensing in the stalk-formers was necessary for the cap to be formed. The available evidence suggests that the observed lack of cap-formation is due to the stalk-formers deficiency in DNA-release, but it can not be excluded that other quorum sensing controlled factors in the stalk subpopulation may affect cap-formation. Quorum sensing controls the production of biosurfactants in *P. aeruginosa* (Ochsner and Reiser, 1995), and these amphipathic molecules appears to have multiple roles in *P. aeruginosa* biofilm development, one of which is to facilitate surface associated bacterial migration and thereby the formation of mushroom caps (Pamp and Tolker-Nielsen, 2007). However, we find it unlikely that the *P. aeruginosa pilAlasRrhlR* mutants deficiency in biosurfactant production could be the cause of the observed phenotype in the wild type/*pilAlasRrhlR* mixed biofilm where the wild type, i.e. the potential cap-former, is able to produce biosurfactants.

The factors involved in coordinating the formation of the mushroom-shaped structures in *P. aeruginosa* biofilms has been investigated using computer based modelling (Picioreanu *et al*., 2007). In support of a coordinating role of matrix components in the development of the mushroom caps, the computer simulations predicted that if motility and chemotaxis were the only factors in operation then vertical strings of cells would be formed in the biofilms (Picioreanu *et al*., 2007).

In summary the present work suggests that the formation of the mushroom-shaped structures in *P. aeruginosa* biofilms occurs in a developmental process involving formation of initial microcolonies (stalks) by a non-motile subpopulation that releases extracellular DNA, and subsequently become capped by a motile subpopulation that uses flagella for surface associated motility and type IV pili for binding to the extracellular DNA.

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Paper 5

Pamp, S. J., Haagensen, J.A.J., Molin, S., and Tolker-Nielsen, T. A Distinct Subpopulation of Cells in *Pseudomonas aeruginosa* Biofilms can be Specifically Eradicated Using Antimicrobial Compounds that Cause Membrane Disruption.

This manuscript is in preparation and represents work in progress. It is set up as a two column layout for lucidity purposes.

A Distinct Subpopulation of Cells in *Pseudomonas aeruginosa* Biofilms Can be Specifically Eradicated Using Compounds that Cause Membrane Disruption.

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Biofilms are reported to be inherently refractory towards antimicrobial attack and therefore might contribute to the persistence of infections. *Pseudomonas aeruginosa* biofilms with mushroom-like shaped multicellular structures consist of two phenotypic subpopulations, the cap-forming and the stalk-forming subpopulation. Recent data demonstrate that the cap-forming subpopulation is composed of cells exhibiting high metabolic activity, and that these cells specifically can be killed by antimicrobial agents that interfere with metabolic processes such as replication and translation. In contrast, the stalk-forming subpopulation is composed of cells exhibiting low metabolic activity and is refractory to the antimicrobial agents that are able to kill the cap-forming subpopulation. Here we demonstrate that the stalk-forming subpopulation exhibits increased susceptibility towards membrane-targeting compounds. In most cases, concentrations equivalent to the minimal inhibitory concentration (MIC)-value of a particular membrane-targeting compound were sufficient to induce cell death in the stalk-forming subpopulation. In contrast to the stalk-forming subpopulation, the cap-forming subpopulation exhibited phenotypic tolerance to membrane-targeting compounds. Our data suggest that the cap-forming subpopulation is able to adapt to exposure with membrane-targeting agents involving different genetic determinants, dependent on the membrane-targeting compound used.

Introduction

The majority of bacteria on Earth are assumed to live in multicellular communities, also referred to as biofilms. Occasionally, biofilms are harmful to humans as they can be the cause of persistent infections (Costerton *et al*., 1999; Høiby *et al*., 2001; Hall-Stoodley, et al., 2004; Hall-Stoodley *et al*., 2006; Parsek and Singh, 2003). These biofilms do persist as they are difficult, and in some cases impossible, to eradicate by conventional anti-microbial therapy (Costerton *et al*., 1999; Fux, et al., 2005; Stewart, 2002; Stewart and Costerton, 2001; Donlan and Costerton, 2002). The reasons and mechanisms underlying this phenomenon are not completely understood and might vary, dependent on intrinsic features of the causative bacteria, prevailing environmental conditions and the antimicrobial compound used (Mah and O'Toole, 2001; Lewis, 2001; Stewart, 2002; Drenkard, 2003; Fux *et al*., 2005). However, evidence is increasing that bacteria living in biofilms are organized into distinct phenotypic subpopulations of cells, and that in fact only certain subpopulations are refractory to a particular antimicrobial compound, whereas the remaining cells exhibit sensitivity (Haagensen, *et al*., 2007; Pamp, *et al*., submitted; Walters, et al., 2003). Therefore, detailed knowledge about the nature and intrinsic characteristics of these distinct subpopulations in biofilms might help to understand antimicrobial tolerance development in biofilms and to develop new treatment strategies to eradicate persistent infections.

Techniques and molecular tools, such as confocal laser scanning microscopy, flow-chamber technology, fluorescent 'tags' (such as Cfp, Gfp, and Yfp) and *in situ* gene expression greatly enhance the possibilities to study, under non-destructive and hydrodynamic conditions, developmental processes, dynamics and differentiations taking place in biofilms. Using combinations of these tools, recent studies discovered that clonal mature *P. aeruginosa* mushroom-like structured biofilms are composed of at least two confined subpopulations of cells, namely a stalk-forming and

a cap-forming subpopulation, each exhibiting a distinct phenotype, e.g. regarding metabolic/physiological activity, gene expression, and production of matrix components (e.g. Allesen-Holm, *et al*., 2006; Klausen, *et al*., 2003b; Lequette and Greenberg, 2005; Pamp, *et al*., submitted). The development of this mushroom-like structured biofilm appears to depend on a subpopulation of non-motile cells and a subpopulation of motile cells (Klausen, *et al*., 2003b). In early stages of biofilm development the stalk-forming subpopulation is formed by clonal proliferation of non-motile cells. Subsequently the subpopulation of motile cells climbs up on the stalk-forming subpopulation to establish as the cap-forming subpopulation. Factors that facilitate cellular migration by the motile subpopulation of cells have been identified as flagella, biosurfactants, type IV pili and extracellular DNA (Allesen-Holm, *et al*., 2006; Barken, *et al*. in prep; Klausen, *et al*., 2003b; Pamp and Tolker-Nielsen, 2007). Moreover, other factors such as quorum sensing, polysaccharide synthesis, chemotaxis, and nutritional conditions, have been found to impact on the formation of these two subpopulations in *P. aeruginosa* biofilms (e.g. Barken, *et al*., in prep.; Jackson, *et al*., 2004; Klausen, *et al*., 2003a; Matsukawa and Greenberg, 2004; Shrout, *et al*., 2006).

Recent observations provide evidence, that the stalk-forming and cap-forming subpopulations exhibit differential susceptibility phenotypes towards various antimicrobial agents. Whereas the cap-forming subpopulation exhibits sensitivity to conventional antimicrobial compounds, such as ciprofloxacin, tetracycline, and tobramycin, the stalk-forming subpopulation survives these treatments (Henzer *et al*., 2003; Pamp *et al*., submitted). Interestingly, the opposite is true when *P. aeruginosa* biofilms are exposed to either the antimicrobial peptide colistin, the chelator EDTA, or the detergent SDS. These three antimicrobial compounds kill preferentially the stalk-forming subpopulation, whereas the cap-forming subpopulation survives these treatments (Banin, *et al*., 2006; Haagensen, *et al*., 2007; Pamp, *et al*., submitted). Consequently, combined antimicrobial treatments with either ciprofloxacin and colistin, or tetracycline and colistin are able to kill almost all cells in a mature *P. aeruginosa* flow-chamber-grown biofilm by targeting both subpopulations simultaneously (Pamp, *et al.,* submitted).

The fact that conventional antimicrobial agents, which commonly interfere with general vital processes of a cell, such as replication, translation and transcription, preferentially kill the metabolically active cells of the cap-forming subpopulation in a *P. aeruginosa* mature biofilms seems to be plausible. However, it is not entirely clear why compounds such as colistin, EDTA or SDS preferentially kill cells of the stalk-forming subpopulation, and why cells of the cap-forming subpopulation survive these treatments. Regarding colistin treatment recent data provide evidence that the metabolically active cells of the cap-forming subpopulation are able to develop tolerance to colistin by mechanisms involving LPS-modification, mediated by *pmrHFIJKLME*, and antimicrobial efflux, mediated by *mexAB-oprM* (Haagensen, *et al*., 2007; Pamp, *et al*., submitted). In contrast, cells of the stalk-forming subpopulation are not able to develop tolerance to colistin, and are therefore killed. Mushroom-like structured biofilms formed by *P. aeruginosa* strains, which have either a defect in the *pmr*-operon or *mexAB-oprM,* are not able to develop a tolerant cap-forming subpopulation, and both, the *pmr*-operon and *mexAB-oprM* were induced in the cap-forming subpopulation upon colistin exposure (Haagensen, *et al*., 2007; Pamp, *et al*., submitted). However, it is unknown whether tolerance development to EDTA and SDS also require *pmr*-mediated LPS-modification and *mexAB -oprM*-mediated antimicrobial efflux. Moreover, also the factors, which render the cells of the stalk-forming subpopulation sensitive to EDTA, and SDS are unknown by now and these issues will be addressed in this study.

Materials and Methods

Bacterial strains and growth conditions

P. aeruginosa PAO1 wild type tagged with Gfp (Klausen *et al*., 2003a), *P.aeruginosa* PAO1 *pmrF* (mutant ID 35399) tagged with Gfp (Haagensen, *et al.*, 2007), *P. aeruginosa* PAO1 Δ*mexAB-oprM*::Gm^r tagged with Gfp (Pamp, *et al*., submitted), *P.aeruginosa* PAO1 Δ*anr* (Ye *et al.*, 1995), *P.aeruginosa* PAO1 *dnr*::*tet*^{r} (Arai *et al.*, 1995), *P.aeruginosa* PAO1 *narL*::*cat*^{r} (Krieger *et al.*, 2002), *P.aeruginosa* CHA *ihfA*::*tet*^{r} (Delic-Attree *et al.*, 1996), *P.aeruginosa* PAO1 Δ*ptA*::*aacC1-gfp* (Eschbach *et al.*, 2004), and *P.aeruginosa* PAO1 Δ*ptA*::*aacC1-gfp* (Eschbach *et al.*, 2004) were used in this study. To study the expression of the *pmr*-operon and *mexAB-oprM*, *P.*

aeruginosa PAO1 strains harbouring the fluorescent transcriptional reporter fusions P*pmrH-gfp* or p*mexA-gfp* were used (Pamp, *et al*., submitted). For batch cultures of *P. aeruginosa* AB minimal medium (Pamp and Tolker-Nielsen, 2007) supplemented with 1 μM $FeCl_3$ and 10 mM glucose was used. Where appropriate, antibiotics were used for bacterial growth cultures at the following concentrations: gentamycin (Biochrome AG, Germany) at 30 μg/ml, streptomycin (Sigma) at 300 μg/ml, and carbenicillin (Sigma) at 200 μg/ml.

Determination of the Minimal inhibitory concentration (MIC)

The Minimal inhibitory concentrations (MIC) for colistin (*Colimycin*, colistin methanesulfonate; Lundbeck A/S, Denmark), colistin sulfate (Sigma, Germany), EDTA (Ethylenediaminetetraacetic acid tetrasodium salt; VWR BDH Prolab, Germany), SDS (sodium dodecyl sulfate; Sigma, Germany), novispirin G10 (Novozymes A/S, Denmark), and chlorhexidine gluconate (Sigma, Germany) was determined by macrodilution method on aerobic planktonic cultures of *P. aeruginosa* grown in AB minimal medium (Pamp and Tolker-Nielsen, 2007) supplemented with 1 μM $FeCl_3$ and 10 mM glucose.

Cultivation of biofilms

Biofilms were cultivated in flow-cells with individual channel dimensions of 1 x 4 x 40 mm, which were covered with a glass coverslip (Knittel Gläser, Germany). The biofilm flow-cell system was assembled and prepared as described elsewhere (Sternberg and Tolker-Nielsen, 2005). As cultivation medium, AB minimal medium supplemented with 0.3 mM glucose as carbon source was used (Pamp and Tolker-Nielsen, 2007). Individual flow-cells were inoculated with 300 μl aliquots of overnight growth cultures of *P. aeruginosa*, adjusted to an optical density of 0.005 at 500 nm, respectively. Overnight cultures of *P. aeruginosa* were grown in AB minimal medium supplemented with 30 mM glucose under vigorous shaking at 30ºC. To allow the bacterial cells to attach to the substratum, flow-cells were left without flow for 1 hour subsequent to inoculation. Then a laminar flow with a mean flow velocity of 0.2 mm/s was achieved using a Watson Marlow 205S peristaltic pump. The *P. aeruginosa* strain containing plasmid p*mexA-gfp* was cultivated as biofilm without supplementation of carbenicillin, as described previously (Pamp, *et al*., submitted).

Exposure of biofilms to antimicrobial compounds

Biofilms were exposed to the following antimicrobial compounds where indicated: 25 μg/ml colistin, 17 μg/ml EDTA, 0.003% SDS, 20 μg/ml novispirin G10, and 0.002% chlorhexidine digluconate. The colistin used here for biofilm treatment experiments is colistin methanesulfonate, which is a compound undergoing hydrolysis in aqueous solutions to form the active compound colistin sulfate (CS) in a time-dependent manner (Bergen, *et al*., 2006). On colistin (colistin methanesulfonate) is focused in this study, since this is the actual compound, which is mainly used in medical settings for treatment of infections, in particular for treatment of *P. aeruginosa* lung infections in cystic fibrosis patients (Høiby *et al.*, 2005; Littlewood *et al*., 2000; Li *et al*., 2006). Treatment of biofilms with antimicrobial compounds was achieved by supplementing biofilm cultivation media with the required antimicrobial compounds at the appropriate final concentrations and addition of the fluorescent dead-cell indicator propidium idodide (Sigma, Germany) at a final concentration of 0.3 μM. In non-treated control experiments only 0.3 μM propidium iodide was added to the biofilm cultivation media. Where indicated, 2.5 μM of the fluorescent indicator dye Syto 9 (Molecular Probes) was added 15 minutes prior to image acquisition, to counterstain for live biofilm cells.

Microscopy and image processing

Image acquisition was performed with a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Germany) equipped with an argon and a NeHe laser and detectors and filter sets for simultaneous monitoring of Gfp and Syto 9 (excitation, 488 nm; emission, 517 nm) and propidium iodide (excitation, 543 nm; emission, 565 nm). Images were obtained using a 40x/1.3 Plan-Neofluar oil objective. Simulated multichannel cross-sections were generated using Imaris software package (Bitplane AG, Switzerland).

Results

We recently observed that the stalk-forming subpopulation of mushroom-like structured biofilms of *P. aeruginosa* exhibits sensitivity to the

antimicrobial peptide colistin, whereas the cap-forming subpopulation survives the treatment (Fig. 1A) (Haagensen, *et al*., 2007; Pamp, *et al*., submitted). In addition it was reported that the chelator EDTA and the detergent SDS preferentially kill the stalk-forming subpopulation, whereas the cap-forming subpopulation survives the treatment (Banin, *et al*., 2006; Haagensen, *et al*., 2007). To verify the susceptibility/tolerance phenotype for EDTA and SDS using our experimental setup, we exposed 4-day-grown biofilms of *P. aeruginosa* (Gfp) either to 17 μg/ml EDTA or 0.003% SDS and followed the effect using confocal laser scanning microscopy (CLSM). Both antimicrobial agents killed nearly all cells of the stalk-forming subpopulation in a similar time-dependent manner as colistin (Fig. 1A-C). All cells of the stalk-forming subpopulation were killed within 24 hours of exposure and the overall distribution of live and dead cells did not change during prolonged exposure (data not shown).

The stalk-forming subpopulation of P. aeruginosa biofilms can be killed using membrane-targeting compounds

The fact that colistin, EDTA, and SDS all preferentially kill the stalk-forming subpopulation is striking, and we speculated if the killing of this particular subpopulation by these, although structurally different, compounds might follow a common mechanism. We hypothesized that this phenoltype could be related to the general mechanisms of action of colistin, EDTA, and SDS, since all three compounds are known to disturb the integrity of bacterial membranes (Hancock, 1984; Hancock and Chapple, 1999; Storm *et al.*, 1977; Tamber and Hancock, 2004). To assess if the stalk-forming subpopulation exhibits sensitivity to membrane-targeting compounds in general, or if the observed phenotype is specific for colistin, EDTA, and SDS, we decided to examine the killing effect of two additional compounds, which are known to exert their effects on bacterial membranes, namely the antimicrobial peptide novispirin G10 and the antiseptic chlorhexidine gluconate (Sawai *et al.*, 2000; Steinstraesser *et al.*, 2002; Vitkov *et al.*, 2005). If these compounds also kill specifically the stalk-forming subpopulation, this would strongly support the notion that this cell subpopulation is particularly vulnerable to membrane-targeting compounds, in contrast to the cap-forming cell subpopulation. We exposed 4-day-grown biofilms of *P. aeruginosa* (Gfp) either to 20 μg/ml novispirin G10 or 0.002% chlorhexidine gluconate and followed the effect using CLSM. Indeed, also these two antimicrobial agents killed nearly all cells of the stalk-forming subpopulation in a similar time-dependent manner as colistin, EDTA and SDS (Fig. 1D and E). All cells of the stalk-forming subpopulation were killed within 24 hours of exposure and the overall distribution of live and dead cells did not change during prolonged exposure (data not shown). The cells of the cap-forming subpopulation, which survived the treatment, did not exhibit resistance to the antimicrobial agents used, as examined by plating of harvested biofilm cells on agar containing the particular antimicrobial agent. This suggests that the cells of the cap-forming subpopulation exhibited phenotypic tolerance to the antimicrobial compounds. Altogether, this indicates that the stalk-forming subpopulation of cells in *P. aeruginosa* biofilms exhibits sensitivity to membrane-targeting antimicrobial compounds, whereas the cap-forming subpopulation exhibits tolerance.

The stalk-forming subpopulation exhibits susceptibility to concentrations of membrane-targeting compounds, which are equivalent to their respective MIC-value

When we examined the effect of novispirin G10 and chlorhexidine gluconate on *P. aeruginosa* we recognized that concentrations nearly equivalent to the minimal inhibitory concentration (MIC) of the particular antimicrobial compound were sufficient to induce cell death in the stalk-forming cell subpopulation. The MIC for novispirin G10 was determined with 30 μg/ml, whereas only 20 μg/ml (0.67xMIC) novispirin G10 were sufficient to kill the cells of the stalk-part (Fig. 1D, and Table 1). This was surprising, as biofilms are often described to be relatively unaffected towards MIC-equivalent concentrations of conventional antimicrobial compounds. A similar effect was observed for chlorhexidine gluconate, as the MIC for this antimicrobial compound was 0.0008%, and the concentration sufficient to kill almost all cells of the biofilm cells in the stalk part was 0.001% (1.25x MIC) (Fig. 1E, and Table 1). With respect to SDS, accurate MIC-determinations were difficult, as *P. aeruginosa* was able to grow in the presence of relatively high concentrations of SDS, which is consistent with previous reports (Klebensberger *et*

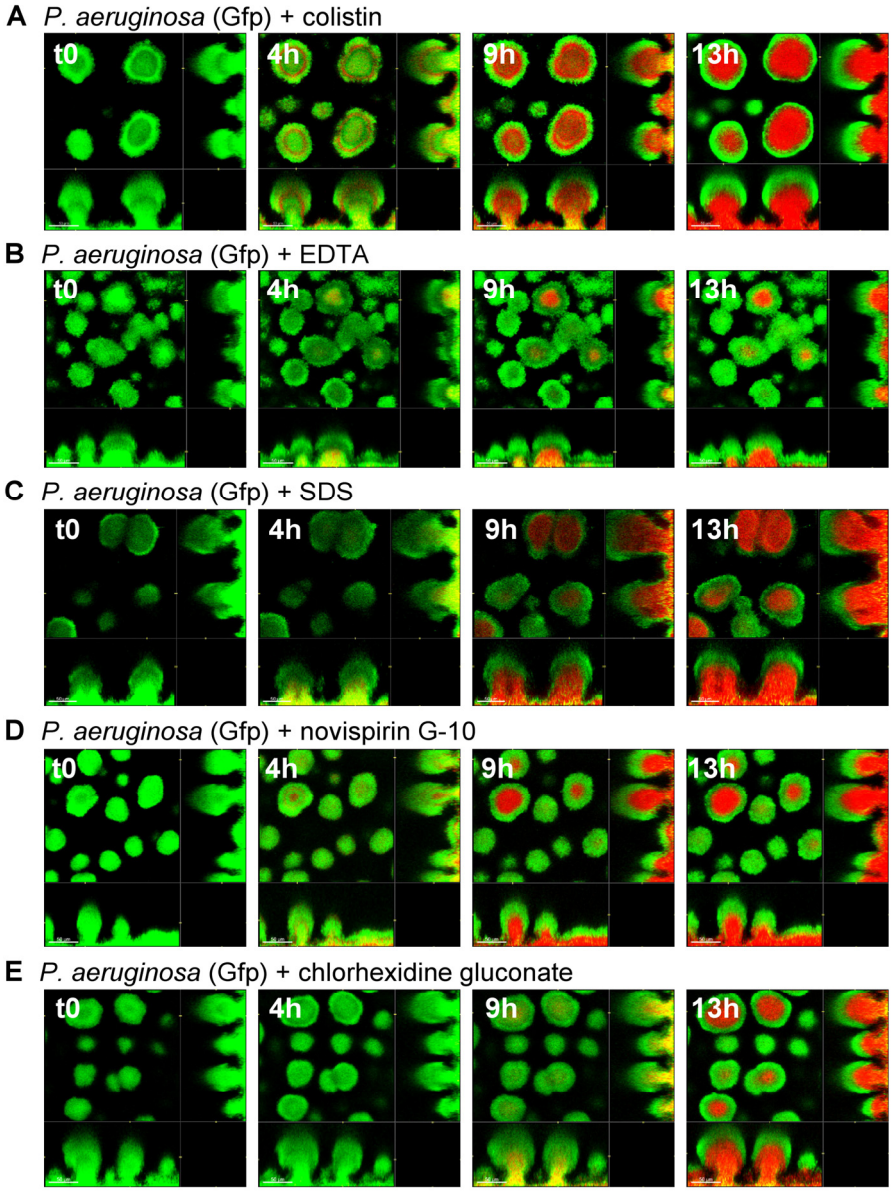

Fig. 1. Spatio-temporal distribution of live and dead cells in *P. aeruginosa* biofilms treated with membrane-targeting antimicrobial agents. Biofilms of *P. aeruginosa* PAO1 (Gfp) were grown for 4 days and then continuously exposed to either 25 μg/ml colistin (A), 17 mg/ml EDTA (B), 0.003% SDS (C), 20 μg/ml novispirin G10 (D), or 0.001% chlorhexidine gluconate (E) in the presence of the dead cell indicator propidium iodide. Confocal laser scanning micrographs were acquired at time point t_0 (prior to exposure) and 4, 9, and 13 hours subsequent to the beginning of treatment. The images show a horizontal section with two flanking images representing sections in the xz and yz planes, respectively. Live cells appear green due to constitutive expression of Gfp and dead cells appear red, due to staining with the dead cell indicator propidium iodide. Scale bar 40 μm.

al., 2006; Klebensberger *et al.*, 2007). We found that growth in the presence of SDS was significantly inhibited at concentrations of 0.6%, whereas only 0.003% SDS (0.005xMIC) were required to kill the cells of the stalk-forming subpopulations in biofilms (Fig. 1C, and Table 1). A concentration of EDTA equivalent to the MIC-value for this compound (MIC_{EDTA} 0.1 μM) by contrast was not sufficient to kill the biofilm cells of the stalk-part, as 17 mM were required to kill the stalk-forming subpopulation within the timeframe of the experiment. To be able to determine the effect of MIC-equivalent concentrations of colistin in killing of the stalk-forming subpopulation of biofilm cells in a comparable manner, we examined the effect of colistin sulfate (CS), as this is the antimicrobial active form generated by hydrolysis of the prodrug colistin (Bergen, *et al.*, 2006). The MIC for colistin

Table 1. Minimal inhibitory concentrations (MIC) and concentrations used to induce cell death in the stalk-forming subpopulation of *P. aeruginosa* biofilm cells.

Antimicrobial agent	Minimal inhibitory concentration (MIC)	Concentration used to kill the stalk-forming biofilm cells
colistin sulfate[a]	0.8 μg/ml	0.8 μg/ml
EDTA	0.1 μM	17 mM
SDS	0.6%	0.003%
novispirin G10	30 μg/ml	20 μg/ml
chlorhexidine gluconate	0.0008%	0.001%

[a]Colistin sulfate (also referred to as 'colistin' in other studies) exhibits antimicrobial activity, and is generated by hydrolysis of the prodrug colistin (colistin methanesulfonate) (Bergen *et al.*, 2006).

sulfate was 0.8 μg/ml (Table 1), and this concentration was also sufficient to kill all cells in the stalk within 24 hours of exposure (data not shown). If colistin (colistin methanesulfonate) was used for MIC determinations, instead of colistin sulfate, a slightly higher value (2.5 μg/ml) was achieved, most likely because colistin needs to undergo hydrolysis into colistin sulfate before exerting its antimicrobial activity. Exposure of *P. aeruginosa* biofilms to MIC-equivalent concentrations of conventional antimicrobial compounds, such as ciprofloxacin or tetracycline, did not induce cell death in any of the biofilm cells (data not shown). Altogether the observations indicate that for most membrane-targeting compounds used in this study, MIC-equivalent concentrations were sufficient to kill the stalk-forming biofilm cells of the *P. aeruginosa* biofilm. However, the cap-forming subpopulation survived the treatment by these compounds.

The pmr-operon is not required for tolerance-development of the cap-forming subpopulation to EDTA

In contrast to the stalk-forming subpopulation, the cap-forming subpopulation survives treatment with EDTA. Development of tolerance of the cap-forming cell subpopulation to colistin in *P. aeruginosa* biofilms was recently found to depend on the *pmr*-operon and a link between the *pmr*-operon and resistance to the chelator EGTA was recently observed for *Salmonella* (Haagensen, *et al*., 2007; Murray, *et al*., 2007; Pamp *et al.*, submitted). EDTA is known to chelate divalent cations such as Mg^{2+}, and Mg^{2+}-limitation is known to induce expression of the *pmr*-operon in *P. aeruginosa* (McPhee, *et al*., 2003). To examine if the *pmr*-operon is induced in *P. aeruginosa* biofilms upon EDTA exposure, which might indicate its possible involvement in tolerance development to EDTA, we exposed 4-day-grown biofilms of *P. aeruginosa* harbouring the fluorescent reporter fusion *pmrH-gfp* to 17 μg/ml EDTA in the presence of the dead cell indicator propidium iodide and followed the effect using CLSM. An induction of the *pmr*-operon in the cap-part of the biofilm was observed within 4 hours of exposure (Fig. 2A). The cells of the stalk-forming subpopulation, which did not exhibit an induction of the *pmr*-operon were killed by EDTA subsequently. The same phenotype was recently observed in biofilms of *P. aeruginosa pmrH-gfp* when exposed to colistin (Pamp *et al.*, submitted). As biofilms formed by *pmr*-mutants of *P. aeruginosa* are not able to develop a colistin tolerant subpopulation (Haagensen, *et al*., 2007; Pamp, *et al*., submitted), we examined sensitivity of a *pmrF*-mutant to EDTA. Surprisingly, the *pmrF*-mutant did not exhibited increased sensitivity to EDTA, instead this strain exhibited wild type phenotype (Fig. 2B and Fig. 1B). Also a biofilm formed by a *pmrB*-mutant exhibited wild type phenotype when exposed to EDTA (data not shown). This indicates, that although EDTA can induce expression of the *pmr*-operon in the cap-forming subpopulation, induction of this LPS-modification system does not confer tolerance of the cap-forming subpopulation to EDTA.

The pmr-operon is not required for tolerance development of the cap-forming subpopulation to SDS

To investigate if the *pmr*-operon is induced in the cap-forming subpopulation upon exposure to the detergent SDS, we exposed 4-day-grown biofilms of *P. aeruginosa pmrH-gfp* to 0.003% SDS in the presence of propidium iodide and followed the effect by CLSM. As can be seen in Fig. 3A, the *pmr*-operon is not induced upon exposure of the biofilm to SDS. Cells of the stalk-forming subpopulation are killed by SDS-treatment, and the surviving cells of the cap-forming subpopulation can be visualized

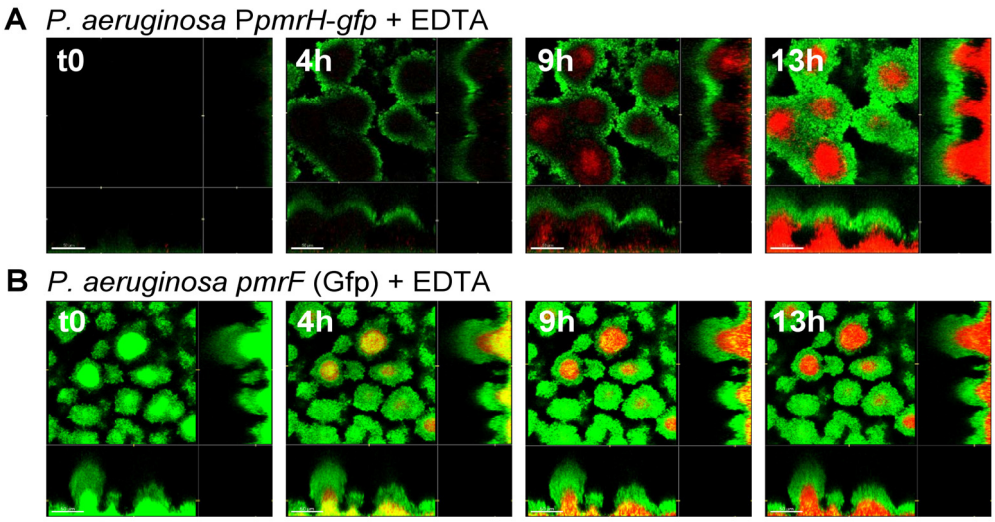

Fig. 2. The *pmr*-operon is expressed upon EDTA exposure but not required for tolerance development to EDTA in *P. aeruginosa* biofilms. Biofilms of *P. aeruginosa* PAO1 Tn7-P*pmrH-gfp* (A) and *P. aeruginosa* PAO1 *pmrF* (Gfp) (B) were grown for 4 days and then continuously exposed to 17 μg/ml EDTA. Confocal laser scanning micrographs were acquired at time point t_0 (prior to exposure) and 4, 9, and 13 hours subsequent to the beginning of treatment. The images represent vertical sections of biofilms, respectively. Live cells appear green due to inducing expression of the *pmr*-operon (A) or constitutively expression of Gfp (B) and dead cells appear red, due to staining with the dead cell indicator propidium iodide. Scale bar 40 μm.

with the fluorescent stain Syto 9. According to this, a *pmr*-mutant should not exhibit an increased sensitivity to SDS in biofilms compared to the wild type. Indeed, when we exposed a 4-day-grown biofilm formed by *P. aeruginosa pmrF* to 0.003% SDS the same phenotype was observed as for the wild type, namely sensitivity of the stalk-forming subpopulation to SDS and tolerance of the cap-forming subpopulation (Fig. 3B and 1C). Furthermore, a biofilm formed by a *pmrB*-mutant did not exhibit increased sensitivity to SDS than the wild type (data not shown). This indicates that the *pmr*-operon is not involved in tolerance development of the cap-forming subpopulation to SDS.

Discussion

Biofilms are said to be inherently refractory towards antimicrobial attack and therefore might contribute to the persistence of infections. Recent observations on *P. aeruginosa* biofilms indicate that these multicellular structures consist of two distinct phenotypic subpopulations and that in fact only one of these subpopulations is refractory towards a particular antimicrobial agent, whereas the other subpopulation exhibits susceptibility to the same agent (e.g. Haagensen *et al.*, 2007; Pamp *et al.*, submitted). Therefore detailed knowledge about the characteristic traits of these distinct subpopulations and the mechanisms involved in tolerance development to antimicrobial agents in biofilms might help to unravel the role of the bacterial biofilm mode of life in relation to the persistence of infectious diseases.

Recent studies observed that when *P. aeruginosa* mushroom-like structured biofilms are exposed either to colistin, EDTA, or SDS, the stalk-forming cell subpopulation is killed, whereas the cap-forming cell subpopulation survives the treatment. Here we show that the same spatial distribution of live and dead cells can be observed when *P. aeruginosa* mushroom-like structured biofilms are exposed to the antimicrobial peptide novispirin G10 or the antiseptic chlorhexidine gluconate. We conclude that the observed susceptibility/tolerance phenotype might be related to the mechanism of action of these antimicrobial compounds, as all compounds exert their effects on interference with the function of bacterial membranes. Interestingly, it appears as that relatively low concentrations of the respective antimicrobial agents, similar to their MIC-value, are sufficient to induce cell death in the stalk-forming subpopulation of biofilm cells. Only MIC-equivalent concentrations of EDTA are not able to induce cell death in the stalk-forming subpopulation. The reason might be in difficulties in determination of a precise MIC-value for EDTA, as EDTA is likely to chelate the iron ions in the growth medium, and as iron is essential for growth, *P. aeruginosa* might therefore not be able to grow in the presence of the amounts of EDTA used. Nevertheless, the fact that relatively low concentra-

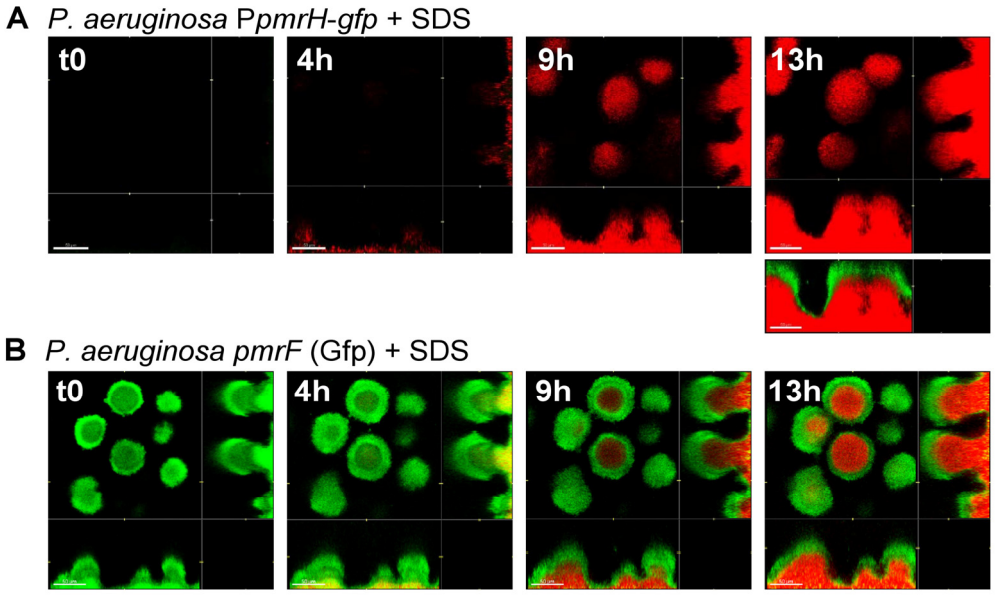

Fig. 3. The *pmr*-operon is not required for tolerance development to SDS in *P. aeruginosa* biofilms. Biofilms of *P. aeruginosa* PAO1 Tn7-P*pmrH-gfp* (A) and *P. aeruginosa* PAO1 *pmrF* (Gfp) (B) were grown for 4 days and then continuously exposed to 0.0003% SDS. Confocal laser scanning micrographs were acquired at time point t_0 (prior to exposure) and 4, 9, and 13 hours subsequent to the beginning of treatment. The images represent vertical sections of biofilms, respectively. Live cells appear green due to staining with the fuorescent dye Syto 9 (A) or constitutively expression of Gfp (B) and dead cells appear red, due to staining with the dead cell indicator propidium iodide. Scale bar 40 μm.

tions of colistin sulfate, SDS, novispirin and chlorhexidine gluconate are able to kill the stalk-forming subpopulation of cells suggests that these biofilm cells are particularly sensitive to membrane targeting antimicrobial agents. This is supported by the finding that in comparison to the stalk-forming biofilm cells, MIC-equivalent concentrations of e.g. colistin sulfate are not able to reduce the number of cells in exponential or stationary phase planktonic cell populations (data not shown).

Our recent study, aimed at understanding tolerance-development to colistin in *P. aeruginosa* biofilms, provides evidence that the prevailing metabolic/ physiological state of the two biofilm cell-subpopulations impact on the spatial distribution of live and dead cells upon exposure with various antimicrobial compounds (Pamp *et al.*, submitted). The cells of the cap-forming subpopulation exhibited high metabolic activity and were able to develop tolerance to colistin, whereas the cells of the stalk-forming subpopulation exhibiting low metabolic activity were not able to develop tolerance, and hence were killed by colistin (Pamp *et al.*, submitted). This could suggest that the growth state of *P. aeruginosa* cells *per se* might impact on antimicrobial susceptibility and ability to develop tolerance to colistin and other membrane-targeting compounds. However, experiments aimed at examining the role of the bacterial growth activity on susceptibility to membrane targeting compounds, by involving exponential and stationary phase planktonic cell populations, could not support this hypothesis, as no difference in the killing-rate between the two planktonic cell populations was observed (data not shown). Alternatively, factors related to anaerobic conditions, but independent of the actual growth rate, might impact on the increased sensitivity of the stalk-forming subpopulation to the membrane-targeting com-pounds. Although so far, no direct measurements have been undertaken for the flow-chamber system used here, that demonstrate that anaerobic conditions are prevailing in the stalk of the mushroom-like structure, it is strongly assumed that the concentration of dissolved oxygen is low in these areas of the biofilm. Oxygen profiles have been measured in a similar flow-chamber system and provided evidence that oxygen is limited in the deeper areas of the multicellular structures (DeBeer *et al.*, 1994). Moreover, induction of *nirS* gene expression (required for anaerobic respiratory metabolism) in the cells situated in the deeper layers of the multicellular structures, and the presence of reactive nitrogen intermediates (resulting as by-products of anaerobic respiratory metabolism) indicate that low oxygen conditions are prevailing in the deeper areas of *P. aeruginosa* flow-chamber-grown biofilms

(Barraud *et al.*, 2006). To investigate the possible role of anaerobic meta-bolism in susceptibility of the stalk-forming subpopulation towards membrane-targeting com-pounds, we tested various *P. aeruginosa* mutant-strains, affected to different degrees in anaerobic metabolism, in their sensitivity to colistin when grown as biofilms. However, all tested mutants (*anr*-, *dnr*-, *narL*-, *pta*-, *ldh*-, and *ihfA*-mutant) exhibited wild type phenotype with respect to susceptibility to colistin in biofilms, namely a colistin-sensitive subpopulation close to the sub-stratum, and a colistin tolerant subpopulation on top (data not shown). Another possibility, that could explain the sensitivity of the stalk-forming cell subpopulation to membrane-targeting compounds, might be that the target of stalk-forming biofilm cells differs from the target of cap-forming biofilm cells. One hypothesis might be that the composition of the membrane of biofilm cells, which are localized in the deeper layers of the biofilm, differs from the composition of the membrane of biofilm cells situated in the upper layers of the biofilm. Although so far no distinct cell subpopulation in biofilms has been assigned to exhibit compositional changes in lipopolysaccharide (LPS), evidence has been provided that the composition of LPS of biofilm-grown cells differs from the LPS composition of planktonic-grown cells (Beveridge *et al.*, 1997; Hunter and Beveridge, 2005). It appears that the relative abundance of A-band and B-band O-antigen varies between these two cell populations, as most notably the amount of B-band LPS is reduced in biofilm-grown cells compared to planktonic-grown cells, resulting in changes of the overall cell surface hydrophobicity (Beveridge *et al.*, 1997; Hunter and Beveridge, 2005). Alternatively, also the relative abundance or com-position of the membrane protein fraction (e.g. efflux pumps, porins) in cells of the stalk-forming subpopulation might differ from cells of the cap-forming subpopulation, and impact on differential uptake or efflux rates of antimicrobial compounds. Investigations are currently ongoing in our laboratory to investigate these issues in relation to the susceptibility phenotype of the stalk-forming cell subpopulation to membrane-targeting compounds.

In contrast to the stalk-forming cell subpopulation, the cap-forming cell subpopulation, exhibits tolerance to the membrane targeting compounds colistin, EDTA, SDS, novispirin G10, and chlorhexidine gluconate. In a recent report we provide evidence that the metabolic active cells of the cap-forming subpopulation are able to develop tolerance to colistin via *pmr*-mediated LPS-modification and *mexAB-oprM*-mediated antimicrobial efflux. It is obvious to assume that *pmr*-mediated LPS modification and/or antimicrobial efflux mediated by the *mexAB-oprM*-genes are likely candidates that might also be involved in adaptation to other membrane targeting compounds. However, our initial experiments aimed at examining the role of the *pmr*-operon and *mexAB-oprM* in tolerance development to these compounds indicated that differential adaptation mechanisms to the single membrane targeting compounds exist. Although the *pmr*-opron is induced in the cap-forming subpopulation of cells upon EDTA-exposure, it is not involved in tolerance development to this compound, as *pmr*-mutants do not exhibit increased sensitivity to EDTA. Also *mexAB-oprM*-mediated antimicrobial efflux is not involved in tolerance development to EDTA, as a *mexAB-oprM*-mutant does not exhibit increased sensitivity to EDTA, and *mexA*-expression is also not induced upon EDTA exposure (data not shown). This suggests, that other determinants must be involved in tolerance development to EDTA. The fact that induction of cell death in the stalk-forming subpopulation by EDTA could be inhibited by the addition of Mg^{2+}, Ca^{2+}, or Fe^{3+} ions (Banin *et al.*, 2006), could suggest that possible prevailing amounts of ions in the cap-part might facilitate some protection of the cap-forming cell subpopulation against EDTA-treatment. However, other determinants are likely to play role as well. Our data concerning the role of the *pmr*-operon in tolerance development of the cap-forming subpopulation to SDS exposure indicate that this LPS-modification system is not involved in tolerance development. A recent study demonstrates that expression of the *mexAB-oprM*-genes from *P. aeruginosa* in a heterologous system confers resistance to a variety of antimicrobial compounds to that strain, among those also SDS (Srikumar *et al.*, 1998). This suggest that tolerance development to SDS might be facilitated by *mexAB-oprM*-mediated antimicrobial efflux. An initial experiment, involving the *mexAB-oprM*-mutant strain *P. aeruginosa* PAO200, showed that a biofilm formed by this strain exhibited an increased sensitivity to SDS during long-term exposure (~30 hours), suggesting that *mexAB-oprM*-mediated efflux might confer some protection towards SDS (data not shown). A recent study aimed at investigating growth of *P. aeruginosa* in the presence of SDS in liquid culture provides data that

suggest that SDS-induced cell-aggregation, facilitated by c-di-GMP-signaling and production of the polysaccharide PSL, confers a survival advantage towards this detergent (Klebensberger *et al.*, 2007). Therefore PSL might also be able to confer protection to SDS in *P. aeruginosa* flow-chamber-grown biofilms. However, to address this issue, a more sophisticated experimental setup is required, as *psl*-mutants are defective in biofilm formation (Jackson *et al.*, 2004; Ma *et al.*, 2006; Matsukawa and Greenberg, 2004), and therefore a proper comparison directly between wild type and *psl*-mutant SDS-treated biofilms is not possible. So far, nothing is known about possible genetic determinants and mechanisms that are involved in tolerance development of the cap-forming cell subpopulation towards the antimicrobial peptide novispirin G10 or the antiseptic chlorhexidine gluconate. Preliminary observations indicate that the *pmr*-LPS-modification system might be involved in tolerance development to novispirin G10, as *pmr*-expression was induced in the cap-forming subpopulation of cells upon novispirin G10 exposure, and a biofilm formed by a *pmr*-mutant exhibited increased sensitivity to this compound. However, this observation needs to be verified. With respect to tolerance development towards chlorhexidine gluconate, a recent study demonstrates that expression of *mexCD-oprJ* is induced upon exposure to chlorhexidine gluconate (Morita *et al.*, 2003). Therefore antimicrobial efflux of chlorhexidine gluconate might be an adaptation strategy that could apply under conditions when *P. aeruginosa* biofilms are exposed to this anti-microbial agent.

Together the results and preliminary observations described here suggest that the confined stalk-forming subpopulation exhibits increased sensitivity towards the membrane-targeting compounds colistin, EDTA, SDS, novispirin G10, and chlorhexidine gluconate. We hypothesize that also other membrane-targeting compounds might specifically kill cells in this area of the biofilm. The mechanism(s) underlying the increased susceptibility of this cell-subpopulation is unclear at present and will be addressed as part of our ongoing investigations. In contrast to the stalk-forming subpopulation the cap-forming subpopulation exhibits tolerance to the membrane-targeting compounds used here. Our data indicate, that *pmr*-mediated LPS-modification and *mexAB-oprM*-mediated anti-microbial efflux might confer tolerance to some, but not all, membrane targeting compounds in *P. aeruginosa* biofilms. Other determinants are obviously involved and the identification of these determinants is part of our present research.

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